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EDITED BY

SIMON FLEXNER, M.D. PEYTON ROUS, M.D.

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BACTERIOSTASIS BY MIXTURE OF DYES.*

By JOHN W. CHURCHMAN, M.D.

(*From the Department of Hygiene of Cornell University Medical College, New York.*)

Plates 1 and 2.

(Received for publication, January 31, 1923.)

The recent observation that acid fuchsin possesses a selective power[1] which is in many respects the opposite of that of gentian violet suggested the advisability of studying the behavior of mixtures of dyes; and the data here presented are the result of that study. Since the time of Ehrlich, workers in the chemotherapeutic field have been largely concerned with efforts to improve antibacterial and antiprotozoal substances by chemical manipulation of the molecule. The observations with acid fuchsin and gentian violet raised the question whether the same end might not be reached in a different way. Might it not be possible by mixing dyes of opposite selective capacities to fortify the weakness of one with the strength of another and thus to obtain a mixture which, in dilutions weaker than that in which either dye alone was effective, would be static for the majority of the commoner bacteria? This question has been answered in the affirmative. It has been found possible to mix two dyes, each of which has been used with a certain success in the treatment of infection, and to obtain a mixture which combines the selective capacities of its components.

Complementary Selective Bacteriostasis.

If a Gram-negative organism like *Bacillus prodigiosus* and a Gram-positive spore bearer like *Bacillus anthracis* be stained with gentian violet the former is unaffected and the latter readily killed. If the experiment be repeated with acid fuchsin the opposite occurs: *Bacillus*

* Aided by a grant from the Committee on Scientific Research of the American Medical Association.

[1] Churchman, J. W., *J. Exp. Med.*, 1923, xxxvii, 1.

prodigiosus is killed and *Bacillus anthracis* unaffected. These experiments have previously been reported in full[1] and the type of selection exhibited by gentian violet has been referred to as normal selective bacteriostasis, that exhibited by acid fuchsin as reverse selective bacteriostasis.

It these two dyes of opposite selective capacities were combined two possibilities as to what would occur were presented. Either a new substance might be formed possessing none of the characteristics of the components, or a mixture might result which combined their selective features. But it proved, on trial, impossible to mix gentian violet and acid fuchsin, for the former was precipitated by contact with the latter. Other mixtures had, therefore, to be sought. It was found that neutral acriflavine possessed a reverse selective bacteriostatic power just as acid fuchsin does. If the experiment described above to test the selective effect of acid fuchsin on *Bacillus prodigiosus* and *Bacillus anthracis* be repeated with acriflavine a similar result is obtained; to this dye, too, the Gram-negative *Bacillus prodigiosus* is much more susceptible than the Gram-positive spore-bearing *Bacillus anthracis*.

These two dyes, gentian violet and acriflavine, will mix, and the resultant mixture combines the selective virtues of its components. It is possible to demonstrate this fact in clear-cut experiments, as illustrated by Figs. 1 to 3. First, the effect of gentian violet alone, acriflavine alone, and a mixture of the two dyes is tested on the Gram-negative *Bacillus pyocyaneus* (Fig. 1). It is seen that gentian violet has no effect on this organism (Fig. 1, *a*), that acriflavine inhibits it markedly (Fig. 1, *b*), and that the mixture kills it completely (Fig. 1, *c*). The experiment is repeated with *Bacillus anthracis* (Fig. 2). This organism is killed by the gentian violet (Fig. 2, *a*), unaffected by acriflavine (Fig. 2, *b*), and killed by the mixture (Fig. 2, *c*).

A similar experiment is then done with a suspension containing both organisms, *Bacillus pyocyaneus* and *Bacillus anthracis* (Fig. 3). This mixed suspension is exposed first to gentian violet; *Bacillus anthracis* is killed and a pure culture of *Bacillus pyocyaneus* results (Fig. 3, *a*). The suspension is next exposed to acriflavine; *Bacillus pyocyaneus* is killed and a pure culture of *Bacillus anthracis* results (Fig. 3, *b*). The suspension is finally exposed to a mixture of the two dyes; both organisms are killed, only an occasional colony appearing on the plate

and these in the strokes made after short exposure to the mixture (Fig. 3, *c*).

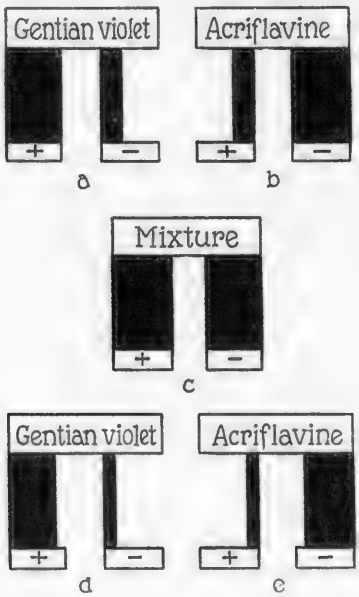
It is clear, from the experiments just described, that gentian violet in a strength sufficient to kill *Bacillus anthracis* may be without effect on *Bacillus pyocyaneus* and that acriflavine in a strength sufficient to kill *Bacillus pyocyaneus* may be without effect on *Bacillus anthracis*. It is also clear that when these two dyes are mixed both organisms are killed by exposure to the mixture. The experiments establish the principle of complementary selective bacteriostasis; they show that certain dyes which possess opposite types of selective bacteriostatic power may be mixed together and that the resulting mixture may possess both types.

Supplementary Selective Bacteriostasis.

While it is evident from what has been said that gentian violet and acriflavine in mixture aid each other in complementary fashion, it is necessary to determine whether they interfere with, or aid each other in the action of each dye on the organism for which it has a selective affinity. In other words, it is still to be ascertained whether the gentian violet interferes with, and weakens the action of acriflavine on *Bacillus pyocyaneus* and whether acriflavine interferes with the action of gentian violet on *Bacillus anthracis*. *Bacillus pyocyaneus* was stained deeply with gentian violet and then exposed to acriflavine. It was found that dilutions of acriflavine which were entirely without effect on unstained organisms readily killed organisms which had been stained with gentian violet. Gentian violet had prepared the way for acriflavine, lessened its task, supplemented its work. There was, in other words, a supplementary selective bacteriostasis.

Two experiments which demonstrate supplementary selective bacteriostasis are shown in Fig. 4. The organism used was *Bacillus pyocyaneus*. On each plate four strokes were made. Stroke *A* was made with organisms which had been exposed to weak acriflavine; growth has been slightly, if at all, impaired. Stroke *B* was made with organisms which had been exposed to gentian violet for 1 hour; the dye was apparently without effect. Strokes *C* and *D* were made with organisms which had been exposed first to gentian violet for 1 hour (like the organisms in Stroke *B*) and then to the same dilution of acriflavine

that was used for Stroke *A*. No growth whatever has occurred on the second plate (Fig. 4, *b*) and only a few scattered colonies have appeared on the first (Fig. 4, *a*). In other words, a strength of acriflavine incapable of killing *Bacillus pyocyaneus* (Stroke *A*) became definitely bactericidal when applied to organisms (Strokes *C* and *D*) which had been previously stained with gentian violet, though the gentian violet alone had been without effect (Stroke *B*). These two dyes, therefore, reinforce each other; in a mixture, so far from interfering with, they supplement each other.



TEXT-FIG. 1, *a* to *e*. (*a*) and (*b*). Complementary selective bacteriostasis. Gentian violet in a dilution lethal for Gram-positives injures somewhat the Gram-negatives (*a*), just as acriflavine in a dilution lethal for Gram-negatives slightly injures Gram-positives (*b*). If the two dyes are mixed the result may be represented by superposing (*a*) on (*b*); *i.e.*, both organisms are killed (*c*).

(*d*) and (*e*). Supplementary selective bacteriostasis. A dilution of gentian violet sublethal for Gram-positives injures very slightly Gram-negatives (*d*), just as a dilution of acriflavine sublethal for Gram-negatives injures very slightly Gram-positives (*e*). If the two dyes in these sublethal doses be mixed the result may be represented by superposing (*d*) on (*e*); *i. e.*, both organisms are killed (*c*).

The facts concerning complementary and supplementary selective bacteriostasis are diagrammatically shown in Text-fig. 1. Text-fig. 1, *a* and *b* represents the facts when gentian violet is used in a strength sufficient to kill the Gram-positive organisms and acriflavine in a strength sufficient to kill the Gram-negative organisms. Death of the organisms is indicated by the broad black band, which with gentian violet completely superimposes the Gram-positive and in the case of acriflavine completely superimposes the Gram-negative. In this strength the gentian violet slightly injures the Gram-negative and the acriflavine slightly injures the Gram-positive (indicated by the partially superimposing black band), an injury which would not be manifest if the organisms were planted on plain agar since they would grow luxuriantly. If the two dyes in this strength be mixed the effect of the mixture would be represented by superimposing Text-fig. 1, *a* on Text-fig. 1, *b*, and Text-fig. 1, *c* would result. This is complementary bacteriostasis.

If we repeat the experiments with weaker dilutions, which alone are inadequate to kill either organism, the result is as shown in Text-fig. 1, *c* and *d*. Gentian violet in this strength has a marked effect on the Gram-positive, though it does not kill it, and a very slight effect on the Gram-negative; acriflavine has a marked effect on the Gram-negative, though it does not kill it, and a very slight effect on the Gram-positive. Neither dye alone kills either organism and the fact that any injury has been done will not be appreciated by the usual cultural experiments since both will grow well after exposure to either dye in this strength. Yet if we expose the organisms to a mixture of the two dyes in this strength the result is as if Text-fig. 1, *a* and Text-fig. 1, *b* were superposed; Text-fig. 1, *c* again results. The slight effect of acriflavine on the Gram-positive organism, supplementing the marked, but not bactericidal, effect of gentian violet, causes its death; and the slight effect of gentian violet on the Gram-negative one, supplementing the marked, but not lethal, effect of acriflavine, causes its death also. Both organisms are killed by the mixture though neither was killed by either dye alone. This is supplementary selective bacteriostasis.

In this study *Bacillus pyocyaneus* was chosen as one experimental organism because previous studies had shown it to be highly resistant to gentian violet though susceptible to acriflavine; and *Bacillus*

anthracis was chosen as the other because the same studies had shown it to be much more susceptible to gentian violet than to acriflavine. There is every reason for supposing that organisms lying between these extremes of resistance and susceptibility will also be more susceptible to the mixture of these two dyes than to either dye alone.

If the two dyes, gentian violet and acriflavine, be mixed together in equal quantities in a mortar and dissolved in water in a strength of 1:2,000 of the mixture, a plum-colored solution results. This solution is absolutely non-irritant for inflamed human mucous membranes (urethral). In laboratory experiments it is bacteriostatic for all the common aerobic organisms. From the clinical tests on a small series of genitourinary infections to which the mixture has been subjected it seems probable that greater success can be obtained from it than from either dye alone. In any event the way has been opened for a new line of attack on the problem of local treatment of infection by selective substances.

SUMMARY.

It is possible to mix two dyes of opposite selective bacteriostatic powers and obtain a mixture which combines the powers of the two components. This possibility has been established by experiments with a mixture of gentian violet, which has a predilection for Gram-positive organisms, and acriflavine, which has a predilection for Gram-negatives; a mixture of these two dyes is quite bactericidal for both groups of bacteria. The weakness of each dye, when used alone, is thus, in the mixture, fortified by the strength of the other.

EXPLANATION OF PLATES.

PLATE 1.

FIG. 1, *a* to *c*. The effect of gentian violet (*a*), neutral acriflavine (*b*), and a mixture of these two dyes (*c*), on *B. pyocyaneus*. Strokes made after exposure of the organisms for 15 minutes (1), 30 minutes (2), 45 minutes (3), and 1 hour (4). The organism is unaffected by gentian violet, greatly injured or killed by acriflavine, and killed by a mixture of the two dyes. Note that the result in (*a*) and (*b*) is the reverse of the result in (*a*) and (*b*) Fig. 2.

FIG. 2, *a* to *c*. The effect of gentian violet (*a*), neutral acriflavine (*b*), and a mixture of these two dyes (*c*), on *B. anthracis*. Strokes made after exposure of the organisms for 15 minutes (1), 30 minutes (2), 45 minutes (3), and 1 hour (4). The

organism is killed by gentian violet, unaffected by acriflavine, and killed by a mixture of the two dyes. Note that the result in (*a*) and (*b*) is the reverse of that in (*a*) and (*b*) Fig. 1.

PLATE 2.

FIG. 3, *a* to *c*. The effect of gentian violet (*a*), neutral acriflavine (*b*), and a mixture of these two dyes (*c*), on a mixed suspension of *B. pyocyaneus* and *B. anthracis*. Strokes made after exposure of the suspension for 15 minutes (1), 30 minutes (2), 45 minutes (3), and 1 hour (4). In (*a*) *B. anthracis* has been killed by the gentian violet and a pure culture of *B. pyocyaneus* results; in (*b*) *B. pyocyaneus* has been killed by the acriflavine and a pure culture of *B. anthracis* results; in (*c*) both organisms have been killed by the mixture of dyes.

FIG. 4, *a* and *b*. Two experiments showing that acriflavine in a strength insufficient, when used alone, to kill *B. pyocyaneus* will kill this organism if it has been previously stained with gentian violet. *A*, organisms which have been exposed to acriflavine; *B*, organisms which have been exposed to gentian violet; *C* and *D*, organisms which have been exposed first to gentian violet and then to acriflavine.

a b c

FIG. 1.

a b c

FIG. 2.

(Churchman: Bacteriostasis by mixture of dyes.)

c b a

FIG. 3.

b a

FIG. 4.

(Churchman: Bacteriostasis by mixture of dyes.)

MUTATION AMONG HOG-CHOLERA BACILLI.

By MARION L. ORCUTT.

(*From the Department of Animal Pathology of The Rockefeller Institute for Medical Research, Princeton, N. J.*)

(Received for publication, February 23, 1923.)

Mutations of various kinds have been demonstrated to occur among bacteria. Among the early workers Baerthlein[1] made an extensive study of many strains including cholera vibrios, typhoid, paratyphoid, hog-cholera, and dysentery bacilli, *B. coli*, *B. enteritidis*, *B. alkaligenes*, *B. prodigiosus*, *B. pyocyaneus*, and streptococci. He described changes or mutations concerning various characters, as colony formation, morphology, cultural reactions, fermentation powers, hemolysis, pigment production, and agglutinability. He also has given a review of much of the work on mutation up to that time. Baerthlein's description of mutation of the *B. suipestifer* (hog-cholera) strains is of special interest in connection with what follows. From hog-cholera cultures he obtained three groups of mutants according to colony formation. He stated that the first appearances of mutation were found after the culture had been kept for a long time (2 to 3 months) on agar without transplanting.

Among the more recent workers, Arkwright[2] described smooth and granular forms among Shiga dysentery cultures which were similar to the hog-cholera types reported in this paper. Arkwright gave quite an extensive review of the earlier literature on mutation. His own work was with the colon-typhoid group and especially with the Shiga bacilli. He reported an S and an R form arising from a single strain which remained constant in weekly subcultures in bouillon. The S form produced smooth colonies, uniform clouding of bouillon, and stable suspension in salt solution. The R form gave granular colonies, grew as a deposit in bouillon, and agglutinated spontaneously in salt solution. He was able to produce specific sera and found the S types of all strains serologically alike but different from the R types and the R forms like each other but different from the S. He found no difference in the virulence of the two types.

Ishii[3] also studied the colon-typhoid group and reported spontaneously and normally agglutinating types. He found connected with these characters smooth colonies and even clouding of bouillon associated with the normally agglutinating

[1] Baerthlein, K., *Arb. k. Gsndhtsamte*, 1912, xl, 433.

[2] Arkwright, J. A., *J. Path. and Bact.*, 1921, xxiv, 36.

[3] Ishii, O., *J. Bact.*, 1922, vii, 71.

type and granular colonies and clumping in bouillon associated with the spontaneously agglutinating type.

De Kruif[4-6] found the smooth and granular types in cultures of the rabbit septicemia bacillus, which he designated D and G types. He showed that the G form was derived from the D. He distinguished it as a mutant by its acid agglutination optimum. He also found a considerable difference in the virulence of these two forms. Apparently the granular type of mutation is widely distributed among various groups of organisms.

In the following pages are described observations made on a number of strains of *Bacillus suipestifer*, the true hog-cholera bacillus, taken from a collection of which many were isolated and tested by Dr. Theobald Smith. Owing to the fact that this subject is not new and that the methods of work have been fairly well standardized most details of the experiments are omitted and only the results briefly given.

Most of the work to be reported was done upon Strain I isolated in 1898 and kept on ordinary nutrient agar. During the course of some agglutination tests this strain was found to agglutinate spontaneously in normal salt solution. A bouillon culture of this strain was clouded and also gave considerable sediment. When it was plated on agar two different types of colonies were found. When cultures were isolated from separate colonies it was found that the even, smooth colony produced uniform clouding in bouillon with no sediment and did not agglutinate spontaneously in normal salt solution, while the more irregular, granular colony grew in bouillon as a sediment and agglutinated spontaneously in salt solution. These two types have been designated A and B; A for the original smooth type and B for the granular mutant.

Later a moist, opaque colony was isolated from the A type culture and designated A-v. Also in an old bouillon culture of the B type mutant a very small colony (B-v) was found which otherwise possessed the B characteristics. These granular and opaque growths in the individual strain are considered mutations since they can be separated from individual colonies into distinct strains which keep

[4] De Kruif, P. H., *J. Exp. Med.*, 1922, xxxv, 561.
[5] De Kruif, P. H., *J. Exp. Med.*, 1921, xxxiii, 773.
[6] De Kruif, P. H., *J. Gen. Physiol.*, 1921–22, iv, 387.

their particular granular or opaque characters through successive transplants.

An examination of twelve other stock cultures consisting of both old and young strains showed the same two types of growth present in seven of these cultures, three of which were rather recently isolated. One older strain gave apparently a pure granular type, while another old culture showed a mixed appearance but the individual types were not separated. In the three remaining strains no granular growth has been detected. This mutation into the granular type does not depend entirely upon the age of the strain but seems to be associated with the length of time a given transplant is kept before it is renewed.

After it was established that two types of growth existed in the hog-cholera strains, experiments were made with Strain I to determine the constancy of each type in pure culture. First the constancy of the types kept on agar and stored in the refrigerator was tested. The second series of experiments was made with bouillon cultures kept at 37°C. and at room temperature. The third group of experiments was made to test the effect of rapid successive passages through bouillon on the constancy of the type. The method of examination was the same as that used by other workers; *i.e.*, at frequent intervals the cultures were streaked on agar plates and the colony formation examined. These observations were controlled by determining the character of the bouillon growth of the individual colonies.

In general, the results indicated that the older A cultures, isolated from a single colony to begin with, if kept long enough without transplanting finally showed B type mutants, but if transplanted often they kept their A form. On the other hand, the mutant B type remained true to type with no reversion, except under the conditions of rapid successive passages through bouillon where it tended to resume some of the original characters. Apparently active growth stimulated A type formation, while aging without transplanting caused mutation to the B type. These observations agree only in part with those of Baerthlein.[1] Whereas he found that his original culture mutated, after standing, into two types, and that these two isolated types both remained true by frequent transplanting, and both changed again on standing, we found that this occurred only with the original culture and its corresponding isolated type (A type),

while with the other mutant (B type) the reverse occurred; *i.e.*, the granular mutant remained true to type when kept 4 to 6 weeks or longer without transplanting but very frequent successive passage through bouillon caused a reappearance of some of the original characters.

Another point mentioned by Baerthlein[7] and De Kruif[4] was an increase followed by a decrease in the number of mutants present in a given culture. This rise and fall in the number of mutants was also observed here with the bouillon cultures of the hog-cholera Strain I kept at 37°C. and at room temperature.

To demonstrate further that B is a mutant with characters distinct from A, the morphology and cultural characters were studied. The behavior in different concentrations of NaCl solution, the acid agglutination, and the behavior in different dilutions of normal serum were tested. Also the virulence of the two types for mice was determined. With the exception of the experiments on virulence, which were carried out with three strains only, these tests were made with the A and B types of six strains and A-v and B-v forms of Strain I. These six strains which have been kept on ordinary agar represent cultures of different ages as indicated by the following dates of isolation: Strains I (1898), II (1921), III (1919), IV (1899), V (1898), and VI (1886).

Description of Types.

Morphology.—The types were similar in form and size. They stained as small, short, Gram-negative rods with a few slightly larger forms often seen in both types. The A type always occurred as single, scattered rods, while the B type usually occurred in clumps. Both types were motile. With Strain I the A type apparently was more active than the B type.

Cultural Characters.—The growth of A and B types on agar and in bouillon has already been described for Strain I and is the same in all the other strains. The A-v mutant was a moist, opaque, non-viscid colony, smooth, glistening, round, and yellowish white. It gave an even clouding in bouillon like the A type. The B-v form

[7] Baerthlein, K., *Centr. Bakt., Ite Abt., Orig.*, 1913, lxxi, 1.

was a small colony, round but more or less granular under a low power. In bouillon it grew as fine suspended particles and sediment.

Salt Solution Agglutination Reactions.—The difference in the reaction of the two types in 0.85 per cent NaCl solution was marked. The A type gave an even, stable suspension while the B type was completely agglutinated, settling out as a sediment and leaving the fluid clear. By diluting the salt solution with distilled water the spontaneous clumping occurred to a smaller degree. The lower the per cent of NaCl the less the clumping of the B type. The same reaction was seen in bouillon. In standard bouillon the B type grew as a sediment with no clouding but if the bouillon was diluted 1:4 with distilled water the B type grew with a fair amount of clouding. This same reaction was reported by Arkwright[7] in his study of the S and R forms of the Shiga dysentery bacilli. Also the A-v and B-v forms agree with the A and B types in the salt solution agglutination reactions.

Acid Agglutination Reactions.—De Kruif[8] considered a change in the acid agglutination optimum to be an index of bacterial mutation. In comparing the acid agglutination reactions of the hog-cholera types it was found that they gave distinctly different titrations. In making the acid tests the pH values were obtained from KH phthalate-NaOH, KH phthalate-HCl, and KCl-HCl mixtures as given by Clark and Lubs.[8] This series gave a long range of pH values. The cultures were grown on agar for 24 hours and the growth suspended in distilled water. The cultures were washed twice and then suspended in a third lot of distilled water and diluted in the usual way to the standard density, 2.4 cm. with the Gates instrument.[9] This washing prevented any spontaneous clumping of the B type. The A type, or original form, gave a low reaction between pH 3.2 and 1.6, but it was never complete. The B type showed a somewhat longer range, between pH 4.0 and 1.0, and a much stronger reaction with complete clumping from pH 3.2 to 1.8. The A-v and B-v forms agree with the A and B types respectively in their acid agglutination reactions. The A types of all strains reacted alike and also the B types of all strains.

[8] Clark, W. M., and Lubs, H. A., *J. Bact.*, 1917, ii, 1.
[9] Gates, F. L., *J. Exp. Med.*, 1920, xxxi, 105.

Normal Agglutination Reactions.—The reactions in normal serum were very different for the two types. An attempt was made to prepare specific A and B sera from rabbits, but it was found that the normal rabbit serum agglutinated the B type to a considerable extent in the case of the two rabbits chosen. To determine whether this was true for normal serum in general, the blood of ten normal rabbits and of three guinea pigs was tested. The tests were made so that in the final dilutions the concentration of the NaCl was only 0.1 per cent; thus the clumping of the B type by salt solution was prevented. The cultures were also washed in distilled water twice before making up to the standard density for the tests. The results showed that normal rabbit serum agglutinated A type only in the low dilutions but agglutinated B type completely at 1:320 or 1:640. With normal guinea pig serum the same was true. Thus normal serum agglutinates B type strongly but A type only slightly. These results again showed the A and B forms as distinct types. However, by agglutinin absorption tests the A and B types are found to be related. A normal rabbit serum which agglutinated A at 1:320 and B at 1:1,280, after absorption with A gave an agglutination reaction negative for A and slight at 1:160 for B and exactly the same results after absorption with B. Thus by absorption of a normal serum with the A type the B agglutinins were also removed, and absorption with the B type removed the A agglutinins as well.

Virulence Tests.—The virulence of A and B types was tested by injecting a 20 hour bouillon growth into mice. Three different strains, Nos. I, II, and III, were used. Strain I is very old, but from the subcutaneous inoculation, although the mice did not die, the culture results indicated a difference in virulence. Cultures were made from the mice by inoculating bits of spleen onto agar slants. The tubes inoculated from mice injected with A type culture all gave positive growth, while some of the tubes inoculated from mice injected with B type culture showed growth but others remained sterile, indicating that the A type culture persisted in the mice while the B type culture was destroyed more readily. By intraperitoneal inoculation of this strain it was found that the A type would kill mice while the same dose of the B type did not. Certain irregularities were noticed but these probably were due to differences in the original

resistance of the mice. With the two younger strains, Nos. II and III, the same difference was indicated in a more definite manner. A given dose (0.2 or 0.05 cc. according to the strain) subcutaneously of A type killed the mice, while the same dose of B type caused no disturbance. Here, too, some irregularities occurred but the differences between the types were striking enough to indicate an appreciable difference in virulence in favor of the A type.

CONCLUSIONS.

1. In the hog-cholera group the mutation from smooth (A) to granular (B) type occurs and a mutant with a moist, opaque colony formation was noted.

2. The B mutant when obtained pure remains a B type without reversion in the stock cultures.

3. The B mutant can be made to assume some of the A characters by rapid successive transplanting in bouillon.

4. The B mutant can usually be found in older transplants of A.

5. The two types may be distinguished by certain cultural characters, by slightly different agglutination reactions, and by different degrees of virulence.

PATHOGENESIS OF THE OCULAR LESIONS PRODUCED BY A DEFICIENCY OF VITAMINE A.*

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Plates 3 to 6.

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The eye lesions developing in animals or human beings as the result of certain types of dietary deficiency have been variously named, keratomalacia, xerophthalmia, and xerosis corneæ. As these names suggest, emphasis has been placed on the corneal changes, and by the majority of investigators the lesion has been assumed to have its origin in the cornea. In harmony with this conception, numerous efforts have been made to demonstrate a preinflammatory degenerative change in this structure. In general, these efforts have proved unsuccessful, although recently Mori[1] has described an initial keratinization, which he attributes to a drying of the eye from insufficient lacrimation.

Clinical observations, particularly at the onset of the condition, led us to question the primary importance of the corneal injury. It was noted, for example, as has been pointed out in a previous paper by one of us,[2] that with the earliest manifestations of the condition in rats—slight photophobia, lacrimation, and reddening of the conjunctivæ—the corneal surface is perfectly smooth with no opacity, or other macroscopic change. After a varying period, the time depending apparently on several factors, including the character of diet, a clouding of the cornea occurs with the formation of the characteristic corneal plaques. These plaques have been generally regarded as

* This investigation was made in cooperation with Dr. Thomas B. Osborne and Dr. Lafayette B. Mendel, under whose direction the feeding of the experimental animals was conducted.

[1] Mori, S., *J. Am. Med. Assn.*, 1922, lxxix, 197.

[2] Yudkin, A. M., *J. Am. Med. Assn.*, 1922, lxxix, 2206.

evidence of complete corneal destruction with consequent ulcer formation. Careful observations, however, have indicated, upon the removal of the plaques, that the epithelium is not entirely lost, and that ulceration of the cornea is a still later phenomenon.

With the object of determining the character and site of origin of the initial lesion, and the sequence of changes leading up to the condition of keratomalacia, a careful histological study was made of the eye and the adjacent structures, including the lids and paraocular glands. These studies, we believe, throw some light on the pathogenesis of the process, and indicate that the common conception of the eye condition as primarily a lesion of the cornea is incorrect.

Method.

Young white rats, from 3 to 4 weeks old and weighing from 45 to 50 gm., were placed on a diet deficient in vitamine A. The composition of the diet was as follows:

Casein	18 gm.
Mineral salts	4 gm.
Starch	54 gm.
Lard	24 gm.
Yeast	0.2 gm.

On this diet, eye symptoms developed after varying periods, but generally after from 30 to 60 days. In order to investigate the ocular lesions at different stages of development, the animals were divided into several groups and sacrificed as follows: (1) immediately upon the appearance of symptoms; and (2) after well marked changes had developed. A series of normal rats was killed as controls. The first series of animals studied was killed by chloroform. In a second series, a guillotine was used in order to eliminate the possibility of changes in the paraocular glands from excessive secretion during anesthesia. The entire head was placed immediately in Zenker's fluid or formaldehyde. After fixation, the orbital structures were dissected out. The eyelids, with a margin of skin, were left attached to the eye and the whole was embedded in paraffin, the lens in each case being first removed through a small opening in the posterior portion of the eyeball

The animals studied were divided clinically into three groups, early, moderately advanced, and advanced, the division being based on the apparent stage of development of the eye condition. While there is obviously no sharp demarcation between the three stages, a brief statement of the symptomatology and gross eye changes will make clear the basis of division.

Symptoms of Eye Involvement.

Usually the first sign of ocular complication is increased lacrimation with moderate photophobia. Shortly afterwards the normally prominent protruding eye tends to recede in its socket, and the photophobia increases. The animal assumes a sleepy appearance. The lacrimation increases but the fluid, at first watery, becomes viscid. The animal rubs the eyes with its front paws, and there is soon a definite edema of the lids. The conjunctival secretion becomes serosanguineous after a few days. The lid hairs fall out and the lids become matted together with dried secretion. In the early stage the cornea shows no visible change, except for marked congestion about its junction with the palpebral conjunctivæ. (There is no visible sclera in the albino eye.) With the progress of the eye disturbance, the cornea shows haziness, particularly about the periphery. The normal corneal reflex disappears. Small yellowish plaques, beginning laterally, spread over its surface and the cornea becomes dry, lusterless, and oily, losing its normal transparency. The plaques can be easily removed. If a solution of fluorescein is introduced after their removal, the drops roll over the surface, leaving it entirely unstained, indicating an absence of ulceration. Examination with a corneal loupe also shows that the surface is intact. If the animal does not succumb, and the lesion progresses, the ulceration of the cornea may take place. The final stage, as has been pointed out in a previous[2] paper, is the picture of keratomalacia, as described by other investigators.

Early Lesions.

Rats killed promptly upon the appearance of the first symptoms of eye disturbance, or within 2 or 3 days thereafter, show practically the same ocular changes histologically. These changes can be most easily described by recording the findings in two typical cases.

No. 484.—A young male rat, weighing 40 gm., was placed on the deficient diet on November 10, 1922. There was a steady gain in weight up to the 39th day of the experiment. On the 38th day, slight lacrimation was noted, with photophobia. On the 40th day, there was a free hemorrhagic secretion from both eyes; on the 41st day, the eyes were almost closed. The rat was killed on the 42nd day; that is, 4 days after the appearance of eye symptoms.

Miscroscopic Examination.—The two eyes show practically the same degree of involvement. In the left eye, there are small focal inflammatory lesions in the nictitating membrane. These consist of an infiltration of the epithelial layer by polynuclear and mononuclear leucocytes, with degenerative changes in the epithelium. Similar lesions are present in the angle of the eye. Both corneæ appear normal. The character of the lesions is well shown in Figs. 1 to 6.

No. 427.—A male rat, 23 days old, weighing 40 gm., was placed on the deficient diet and was killed after 33 days, immediately upon the appearance of signs of eye involvement. It was noticed that the right eye was sunken and that there was a slight hemorrhagic secretion from the same eye. The left eye appeared normal. The animal grew steadily, weighing, when killed, 98 gm.

Microscopic Examination.—The right eye shows an early inflammatory reaction of the conjunctivæ lining both lids. The reaction is most marked in the palpebral fold (Fig. 6). There is keratinization of the superficial layers of the epithelium in the affected areas with an accumulation in the conjunctival sac of keratin shreds, polymorphonuclear leucocytes, red blood cells, and cellular debris. In the ciliary area, there are proliferation of the capillaries and beginning cellular infiltration extending towards the cornea. The cornea itself, however, appears unaltered, except for a slight proliferation of the epidermal cells just opposite the lid lesion. There is neither edema nor multiplication of corneal corpuscles. The left eye presents an almost identical picture. The reaction in the ciliary region is perhaps slightly less advanced.

Both these cases show, in brief, early focal inflammatory lesions in the lid, without recognizable corneal involvement. Only slight deviations from this picture were found in other animals killed at the same stage. In respect to the location of the lesion, however, some variation was observed. The site of election is in the lid, near the palpebral fold. In some instances, foci were seen on the nictitating membrane. Sections through the eye and lids, at different levels, indicate that several foci may be present. Both the upper and lower lids are frequently simultaneously involved; that is, the changes appear equally advanced.

Advanced Changes.

We have classed as moderately advanced the cases in which a beginning corneal involvement is seen, and as advanced those in which a widespread involvement of this structure is found. There is, however, no sharp line of division between the two groups, since one passes over into the other. Detailed descriptions of these advanced changes

are unnecessary, inasmuch as the lesions at this stage have been described in detail by several previous investigators.

The proliferation of the epithelium, with slight keratosis at points just opposite the early lid lesions, appears to be the first change in the cornea (Fig. 1). A lateral ingrowth of the blood vessels from the sclera immediately follows, and in fulminating cases, only a few days elapse before the cornea is markedly edematous, permeated with budding capillaries and proliferating corneal corpuscles, and infiltrated by wandering cells, chiefly of the polymorphonuclear type. In more slowly developing lesions, or in cases of long standing, mononuclear cells may predominate. Marked thickening of the epidermal layer with keratinization and a shelling off of the keratinized epidermis is regularly seen. A feature of the advanced lesion, however, which should be emphasized, is the remarkable vitality of the corneal conjunctiva. The corneal epithelium, as careful clinical observations have indicated, remains intact throughout the early stage, and even with a diffuse inflammatory reaction of the corneal substance, the anterior epithelium may be preserved, as shown in Fig. 7. Occasionally, ulceration develops rapidly, and in the majority of cases, ulcers will appear sooner or later. But in any case, the necrotizing process represents a terminal stage. It is noteworthy that an accumulation of pus may be found in the anterior and posterior chambers, while the corneal conjunctiva is still intact (Fig. 7).

The lid changes in the advanced cases show no such development as those in the cornea. While the focal lesions observed early tend to spread, with sometimes an involvement of the greater part of the palpebral conjunctiva (Fig. 2), there is generally no extension of the infection to the deeper tissues of the lid, and complete sloughing of the epithelium is exceptional. The Meibomian ducts are often conspicuously large (Fig. 6), but it is not certain whether there is a pathological dilatation, since in control animals, similar pictures were occasionally noted. Edema of the lids, noted clinically, is manifest in the microscopic sections. Occasionally an infection of the skin, similar to that in the conjunctiva, is also observed. Hemorrhage into the hair follicle is a common and striking feature. Normally large lymphatic channels are seen within the fibromuscular ring which supports the hair. With the scratching of the eye, these channels are

torn and capillaries are ruptured, with a resulting hemorrhage. Changes in the paraocular glands in advanced cases are described in a separate paper.[3]

The following protocols of two animals showing advanced lesions illustrate very well the changes just described.

No. 377.—A white rat, 28 days old, weighing 50 gm., was placed on the deficient diet. The animal gained in weight until the 45th day of the experiment, when a decline began. The weight then was 71 gm. Symptoms of eye involvement were first noted on the 38th day. There was photophobia, with slight lacrimation, followed within 2 days by a marked injection of the conjunctiva. On the 41st day, the discharge was sanguinous. On the 42nd day, the lids were swollen, with loss of lid hairs. 5 days later, the eyes were completely closed, and on the 52nd day, 14 days after the appearance of eye signs, a plaque was noted on the right cornea. The rat was killed by guillotining, and the head placed immediately in Zenker's fluid.

Microscopic Examination.—Widespread inflammatory reaction is seen in both eyes, involving the entire conjunctivæ and cornea. Both corneæ show a diffuse reaction with numerous blood vessels extending throughout and everywhere an active proliferation of corneal corpuscles. The tissue is diffusely infiltrated with polynuclear and mononuclear cells. The corneal epithelium is thickened with superficial cornification but the layer is everywhere intact.

No. 398.—A rat 28 days old, weighing 50 gm., was placed on the experimental diet October 30, 1922. It began to lose weight on the 22nd day; the weight was then 60 gm. On the 34th day, photophobia was noticed, and on the 35th day, there was a slight hemorrhagic discharge. The next day, the conjunctivæ of the right eye were reddened and the left closed with exudate. On the 42nd day, the hemorrhagic discharge was quite marked, and the lids showed a tendency to stick together. A plaque was noted on the left cornea. The animal was killed and the ocular tissues were fixed for microscopic study.

Microscopic Examination.—An advanced ophthalmia of both eyes with widespread involvement of the corneæ is found. Focal lesions are seen in the lids and nictitating membrane, but in most places, the lesions have become confluent. The plaque noted grossly is made up of necrotic epithelium and exudate. The entire cornea is extremely edematous and infiltrated by blood vessels and numerous wandering cells. On the right side, the anterior chamber is filled with pus.

DISCUSSION.

These studies show clearly the sequence of the changes in the eye resulting from a deficiency of vitamine A. The initial lesion is

[3] Lambert, R. A., and Yudkin, A. M., *J. Exp. Med.*, 1923, xxxviii, 25.

seen in the conjunctiva of the lid. There are generally in both lids multiple foci which tend to coalesce rapidly. As in certain other types of conjunctival infection, a secondary involvement of the cornea quite regularly occurs. The nature of the conjunctival reaction is, as previous investigators have pointed out, essentially that of a bacterial infection. Our studies have not thrown any light on the question as to what determines the initiation of the infection. We have not been able to confirm the observations of Mori[1] that a drying of the eye, with a cornification of the epithelium, antedates the reaction. Indeed, the fact that, in the early stages of the condition, lacrimation is a striking feature cannot be harmonized with Mori's hypothesis. On the other hand, it is possible, as we have suggested in a previous communication,[4] that some of the later phenomena may be due to deficient lacrimal secretion, and consequent drying of the conjunctiva. The fact that as a general rule both eyes are simultaneously involved and that progress in both proceeds at about the same pace is suggestive of a systemic disturbance. This may be conceivably of the nature of a deficient production of antibodies. The recent studies of Smith and Wason[5] lend support to this view. They have shown that, in deficiently fed animals, the bactericidal titer of the serum is distinctly lowered, and there is a less marked reduction in the bacteriotropic activity of the serum. It is also conceivable that there may be protective substances in the secretion of the paraocular glands which diminish as a result of the insufficient diet. In a later paper, changes in one of the paraocular glands will be described which indicate a serious disturbance in its secretion.[3]

SUMMARY.

The earliest lesions in ophthalmia of rats resulting from a deficiency of vitamine A consist in focal inflammatory lesions in the conjunctivæ of the lids and nictitating membrane. The involvement of the cornea, which constitutes the most conspicuous feature of the well developed ophthalmia, is a secondary phenomenon. The characteristic corneal plaque consists of keratinized epithelium beneath which the deeper layers of epithelium are generally found intact.

[4] Yudkin, A. M., and Lambert, R. A., *Proc. Soc. Exp. Biol. and Med.*, 1921–22, xix, 376.

[5] Smith, G. H., and Wason, I. M., *J. Immunol.*, 1923, viii (in press).

Pathologically, the ocular manifestations of a deficiency of vitamine A are referable to a low grade inflammatory process, originating in the palpebral conjunctiva and spreading to the cornea.

The rapidity of development and the degree of destruction probably depend in large part on the type of bacterial infection. The specific changes which lead to the establishment of such an infection have not been determined. It is conceivable that they may be either physiological or anatomical. There is some evidence in favor of a general disturbance of antibody formation in animals on deficient diets. The possibility of a disturbed function in the paraocular glands must be considered.

EXPLANATION OF PLATES.

PLATE 3.

FIG. 1, *a* and *b*. (*a*) Early lesion in the lid conjunctiva of a rat killed after 35 days on the deficient diet. No eye symptoms had been observed. Note the polynuclear infiltration of the mucous glands with the mononuclear reaction in the tissue beneath. There is thickening of the corneal epithelium opposite the lid lesion. (*b*) Higher magnification of the lid lesion.

PLATE 4.

FIG. 2. Widespread reaction in the conjunctiva of the lid with no involvement of the cornea.

FIG. 3. Higher magnification of the lid lesion showing cornification of the epithelium, polynuclear infiltration, dilatation of the blood vessels, and proliferation of the fixed tissue cells in the subconjunctival tissue.

PLATE 5.

FIG. 4. Photomicrograph of low magnification showing (*A*) lids, (*B*) nictitating membrane, (*C*) cornea, and (*D*) iris. An early focal lesion is seen in the conjunctiva of the nictitating membrane.

FIG. 5. Higher magnification of the focal lesion shown in Fig. 4. The infiltrating cells are chiefly polynuclear leucocytes.

PLATE 6.

FIG. 6. Well marked reaction in the conjunctiva involving the conjunctival fold between the lid and nictitating membrane. The space between the folds is filled with cellular debris, pus cells, and keratin shreds. In the lid there is a dilated Meibomian duct.

FIG. 7. Advanced corneal lesion with a widespread vascular reaction and cellular infiltration, but intact epithelium. Note the cellular reaction in the iris with pus cells in the anterior and posterior chambers.

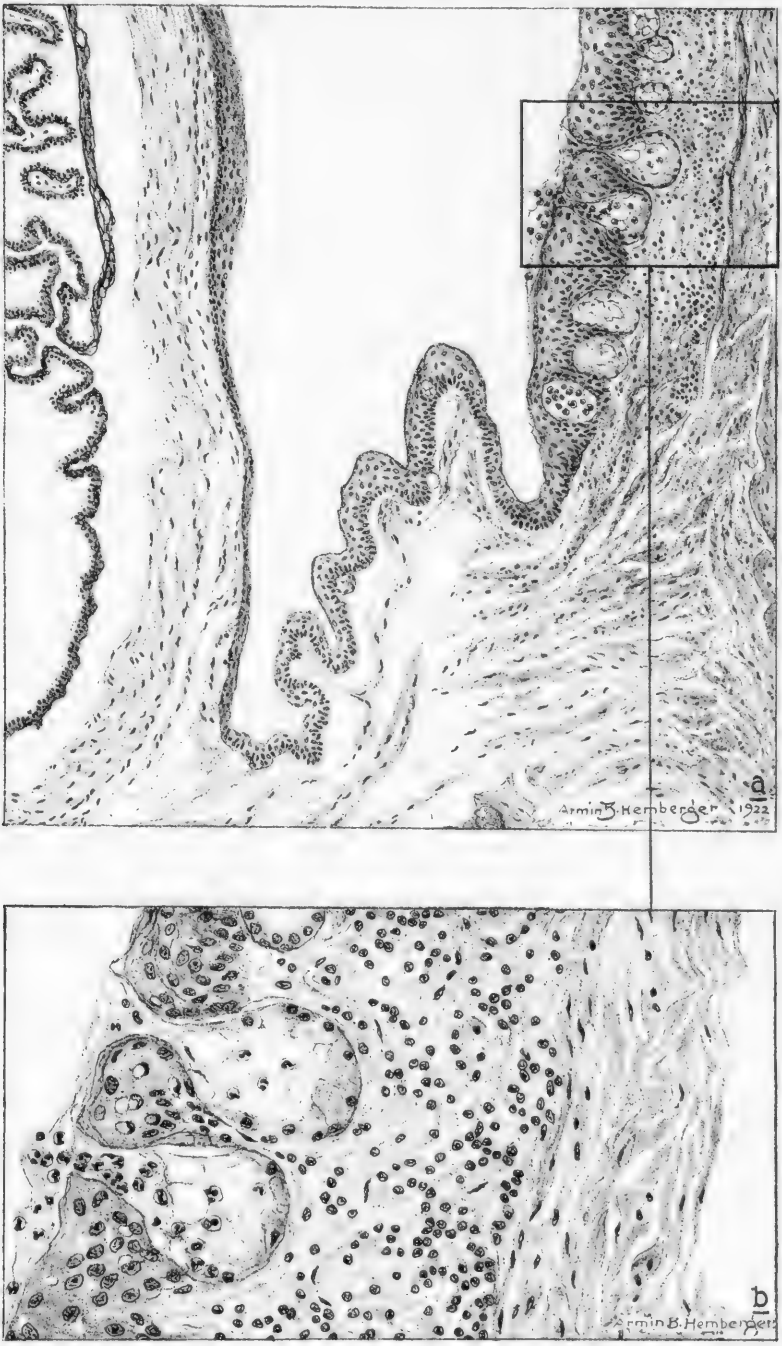

FIG. 1.

(Yudkin and Lambert: Deficiency of vitamine A.)

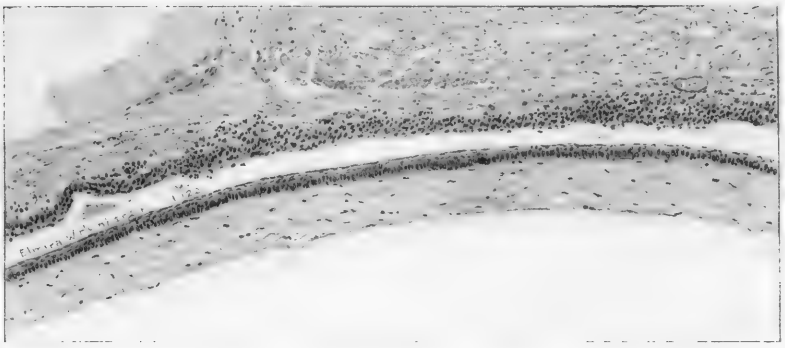

FIG. 2.

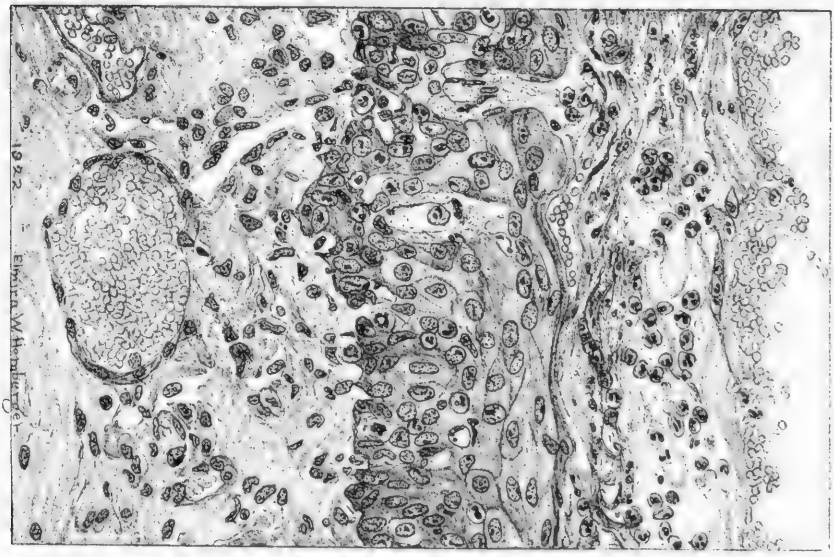

FIG. 3.

(Yudkin and Lambert: Deficiency of vitamine A.)

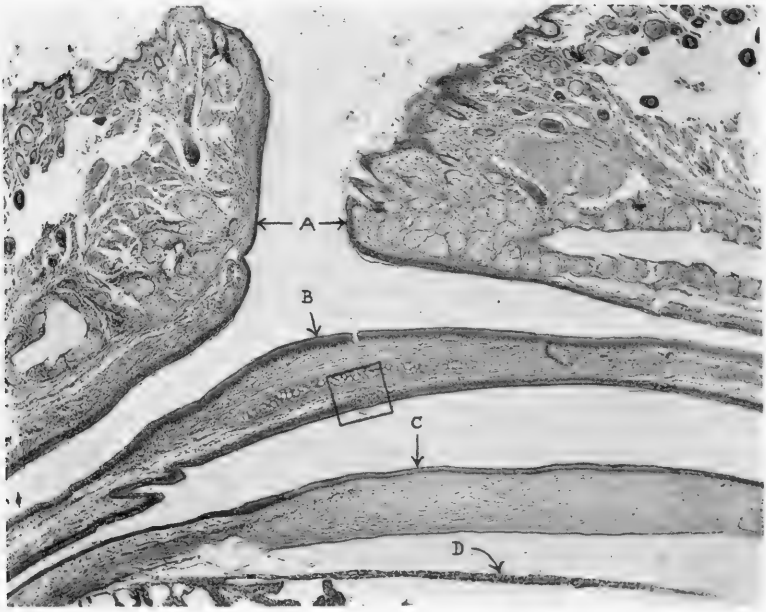

FIG. 4.

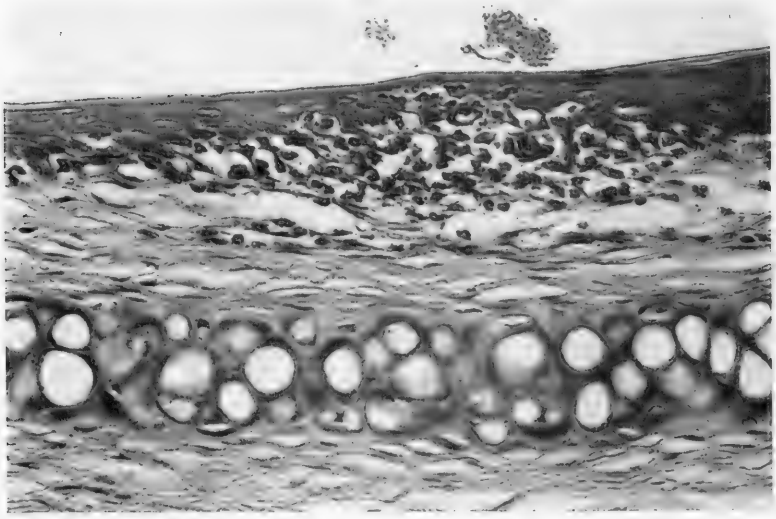

FIG. 5.

(Yudkin and Lambert: Deficiency of vitamine A.)

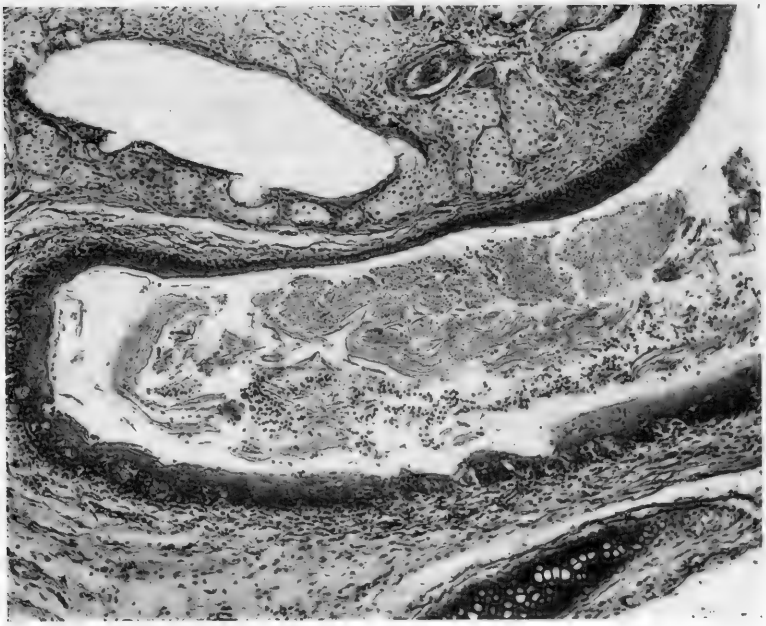

FIG. 6.

FIG. 7.

(Yudkin and Lambert: Deficiency of vitamine A.)

CHANGES IN THE PARAOCULAR GLANDS ACCOMPANYING THE OCULAR LESIONS WHICH RESULT FROM A DEFICIENCY OF VITAMINE A.*

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Plates 7 to 10.

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In ophthalmia produced in rats by diets deficient in vitamine A, lacrimation is an early symptom, whereas later the secretion decreases with a drying of the conjunctiva. This phenomenon suggested the possibility of an involvement of the paraocular glands in this condition. A second observation that led to investigation of the glands was the fact that the lesions resulting from dietary deficiency generally begin simultaneously in both eyes, with relatively little difference in the rapidity of development on the two sides. Although clinically one eye may appear to be unaffected, microscopic investigation shows that unilateral involvement is exceptional. These considerations indicate that while the character of the eye lesion is essentially that of a bacterial infection of the conjunctiva, there is probably some disturbance outside the eye which makes the infection possible. Since it is known that the lacrimal secretion has bactericidal properties, it seems possible that such a disturbance might lie in the activity of the glands that supply the secretion. A careful histological study of the paraocular glands, therefore, appeared warranted. Before presenting the findings, a brief discussion of the anatomy and physiology of these little studied glands may make for clearness.

In the majority of mammals, the conjunctival secretion is supplied by two small organs, the lacrimal and the Harderian glands. There

* This investigation was made in cooperation with Dr. Thomas B. Osborne and Dr. Lafayette B. Mendel, under whose direction the feeding of the experimental animals was conducted.

are, however, in addition, the Meibomian glands, specialized sebaceous glands which lubricate the margins of the lid, and numerous solitary mucous glands scattered through the conjunctiva of the lids.

In certain species, including the rat and mouse, the lacrimal apparatus consists of two parts, one, intraorbital, the other, extraorbital. The former lies generally behind the eyeball, while the latter is found near the angle of the jaw in close relation with the parotid.

TABLE I.

Rat No.	Weight.	
	Body.	Lacrimal and Harderian glands.
	gm.	*mg.*
1	37.5	48.0
2	72.8	118.0
3	80.6	146.0
4	102.0	166.0
5	120.0	170.0
6	150.0	260.0
7	194.0	285.0
8	200.0	257.0
9	205.0	280.0
10	208.0	283.0

The Harderian gland is situated intraorbitally, generally in close apposition to the eyeball. In man, and other primates, it is a small vestigeal structure. In the rat, mouse, and rabbit, and other animals having a well developed nictitating membrane, or third lid, the gland is quite prominent, and in most of these species is considerably larger than the intraorbital lacrimal, with which, in the rat and mouse, it is closely associated. The relatively large size of the two glands is well indicated in Table I, which shows the weight of the body and of the two glands in ten normal rats.

The amount of gland tissue is proportionately far greater than in man, as the following comparison will show. The lacrimal gland in man, according to Canavan,[1] who has recently made a study of the organ in forty cases of mental disease, has an average measurement

[1] Canavan, M. M., *J. Med. Research*, 1922, xliii, 447.

of not more than 2 by 1 by 0.5 cm. This would indicate a weight for the two glands of about 2 gm., or an approximate proportion of gland to body weight of about 1:25,000; whereas in the rat the average proportion, as the figures given in Table I show, is around 1:700, not including the extraorbital lacrimal. In other words, in the rat there is at least thirty times as much glandular tissue supplying secretion for the conjunctivæ as in the human being.

Owing to the close association of the Harderian and lacrimal glands, and the larger size of the former, we confused the two structures in our earlier studies. A review of the literature shows that this mistake has been made before. In Jordan and Ferguson's Text-book of histology, for example, there is a photomicrograph of "the lacrimal gland of the rabbit" which is evidently the Harderian gland.[2] In the rabbit, it may be explained, the two glands are separate and can be easily distinguished grossly, as well as microscopically.

While both glands are acinotubular in type and are similarly lobulated, their component cells are different (Fig. 1). The lacrimal gland is made up of small acini which, at least in fixed preparations, appear to have little or no lumen. The marked shrinkage is, no doubt, the result of the watery character of the cells. The nuclei vary markedly in size and are rich in chromatin. The cytoplasm takes a definitely basophilic stain, like that of pancreatic epithelium. Large vacuoles are numerous.

In the Harderian gland the acini are larger and the cells are uniform in size with relatively small nuclei and abundant eosin-staining cytoplasm. In frozen sections, stained by Sudan III, or Sharlach R, the cytoplasm is seen to be filled with numerous fat granules of varying size. In paraffin preparations, granules obviously of a protein nature, and vacuoles left by the dissolved fat, are numerous. The form of the cells and acini is well shown in Fig. 2.

The exact nature of the secretion of the Harderian gland has not been definitely determined. According to Weber,[3] its function is not essentially different from that of the lacrimal glands, although its

[2] Jordan, H. E., and Ferguson, J. S., A text-book of histology, New York and London, 1916, 681.

[3] Weber, M., Die Säugetiere; Einfuhrung in die Anatomie und Systematik der rezenten und fossilen Mammalia, Jena, 1904, 140.

secretion is more fatty in character, which is consistent with the fact that this gland is found especially in water mammals as a protection against the water medium, and is lacking in Chiroptera and Simiidæ. Jordan and Ferguson,[2] however, refer to the gland as a mucus-secreting organ. After Zenker fixation, we have observed in the lumina of the acini and ducts a variable quantity of yellow granular precipitate. This is not seen in formaldehyde-fixed tissue. This observation suggests that while fat may be present in the secretion, there are, undoubtedly, other elements of importance.

The paraocular glands were studied microscopically in 42 rats, including 15 with early ophthalmia, 15 with advanced ophthalmia, and 12 controls. The ophthalmia was produced, as described in the preceding paper, by a diet deficient in vitamine A.[4] The rats were carefully observed and were killed at different stages of development of eye lesions.

The controls consisted of six healthy young rats of the same age as the experimental animals, and six old rats suffering from chronic skin and eye infections. The ocular tissues were fixed and sectioned, as previously described, except that in some instances the lacrimal and Harderian glands were dissected free from the eye and sectioned separately. A small part of the Harderian gland, however, was practically always left attached to the eye, to which it is closely bound.

The findings in the Meibomian, lacrimal, and Harderian glands can be best considered separately.

Meibomian Gland.

In a previous communication, describing the conjunctival lesions, we referred to the strikingly large Meibomian ducts,[4] and Mori[5] has reported a similar observation, regarding the change as a cystic dilatation. Since, however, ducts of comparable size have been seen in preparations from control animals, we are not certain that the change is pathological. It is known that the smaller Meibomian ducts unite to form larger ones, and it is probable that the very large channels seen in some sections are collecting ducts. No changes in the glandular epithelium have been demonstrated.

[4] Yudkin, A. M., and Lambert, R. A., *J. Exp. Med.*, 1923, xxxviii, 17.
[5] Mori, S., *Bull. Johns Hopkins Hosp.*, 1922, xxxiii, 357.

Lacrimal Gland.

Only the intraorbital lacrimal has been studied. On account of its small size, it was frequently overlooked in routine sections. It was this fact, as stated above, that led us in the beginning to confuse the lacrimal with the more conspicuous Harderian gland. According to Mori, the lacrimal gland in rats kept on a diet deficient in vitamine A shows a marked shrinkage of the secretory cells of the acini.[5]

"They (the cells) no longer show secretory zones. The nuclei take on irregular shapes, occupy the central part of the cytoplasm, which is very small and stains very poorly. The individual acini of the gland become so small that it is impossible to distinguish them with the low power of the microscope, and the gland appears to be a simple mass of nuclei."

Mori also observed in the cells of some of the glands studied large vacuoles which, as specific stains showed, were spaces that had been occupied by fat droplets. The present study confirmed Mori's findings, but the significance of the observations should be questioned in view of the fact that similar histological pictures can be demonstrated in the glands of normal rats. Fig. 1, for example, shows in a gland taken from a normal animal changes which Mori has described. While there is clinical evidence of an altered secretion of the lacrimal gland, particularly in the later stage of dietary ophthalmia due to vitamine A deficiency, the histological changes described do not explain the phenomena, for the reason just stated. It is possible that special granule stains may be necessary to demonstrate the change. We have not observed, in the lacrimal gland, definite acute or chronic inflammation, such as is commonly seen in the Harderian gland.

Harderian Gland.

Lesions of the Harderian gland were found associated with both early and advanced ophthalmia, but the incidence of involvement was highest in rats showing advanced conjunctival changes. In only one instance were marked lesions seen in a healthy control. On the other hand, in one of the diseased controls, an old animal with skin and eye infection, the gland showed striking lesions.

Degenerative Changes.—The Harderian gland has been so little studied by pathologists that the normal variation in the form and staining properties of the acinar epithelium is not known. In some of the control specimens, a definite granulation and vacuolation of the cytoplasm were observed, while in many of the experimental animals, particularly those showing advanced ophthalmia, extreme vacuolation and an apparent disintegration of the cytoplasm were noted. This change is well shown in Fig. 3. In one gland, widespread necrosis of the epithelium was seen with only a slight, apparently beginning, inflammatory reaction, a lesion comparable to the necrosis of the renal epithelium in mercury or uranium nitrate poisoning (Fig. 4).

In general, we believe that the data are insufficient as yet to warrant a statement as to just what constitutes a degenerative change. It is possible that swelling, granulation, and vacuolation may represent variations in functional activity. A marked dilatation of the acini constitutes the most striking alteration in several preparations. In some, the change was diffuse (Fig. 5), but more often only a single lobule was affected.

Acute Inflammatory Changes.—In five of the glands studied an acute inflammatory reaction was found. The histological features of the reaction did not differ essentially from those seen in other glandular organs. Polymorphonuclear leucocytes were abundant, often filling the acini and widely infiltrating the interacinar tissue. In some instances, the lesions were focal, involving only one or a small group of lobules, but in many cases the entire gland was involved. In the majority the lesion was bilateral. The involvement of the ducts was a conspicuous feature in practically all specimens. In some the ducts were markedly dilated, and the lumen was filled with polymorphonuclear leucocytes and cellular debris (Fig. 6.) Abscess formation was the exception, rather than the rule, being seen in only two instances.

Chronic Inflammatory Changes.—The transition from acute to chronic lesions was not sharp, and as in other organs, there was frequently seen what might be termed a combination of acute and chronic inflammation; that is, the process was quite active, with numerous polymorphonuclear leucocytes, but associated with this

was evidence of healing, with granulation tissue and well developed scar tissue, and numerous phagocytic mononuclear cells. In two instances, the lesions were practically healed, leaving a scarred, atrophic gland. Figs. 7 and 8 show the varied picture which may result from the chronic inflammatory process. There is quite as much variation in the pathology of the gland as can be demonstrated in regularly studied organs, like the pancreas or thyroid.

Lymphoid Cell Accumulations.—In five rats, two of which were normal, discrete foci of lymphoid cells were found scattered through the glands. These nodules varied in size from a collection of a dozen cells to patches occupying a space equal to that of a group of acini. In one gland, three nodules were found that corresponded in every particular with those described by Canavan[1] in the human lacrimal gland. The accumulations probably have no significance, and are to be classed with accumulations in the wall of the bronchi, kidney cortex, and other situations. In the present work, they were certainly not definitely related to the eye infection.

SUMMARY.

There is evidence suggesting that the ophthalmia which results from a deficiency of vitamine A is secondary to some change outside the eyes, and that this change may lie in the paraocular glands. In rats these glands consist of the lacrimal, with its watery secretion, the Harderian, with a probable fatty product, and the Meibomian glands. A histological study of these structures shows questionable changes in the lacrimal and Meibomian glands, and marked changes in the Harderian gland.

The changes in the Harderian gland are both degenerative and inflammatory. The degenerative changes consist of swelling, vacuolation, and occasionally complete epithelial disintegration. The inflammatory lesions may be either acute or chronic. The acute reactions are sometimes diffuse, but are more often focal. Definite suppuration occasionally results. The acute process generally passes over into a chronic inflammation with mononuclear cell infiltration, fibrosis, and atrophy. The changes demonstrated indicate a serious disturbance in the secretion of the Harderian gland, such as may conceivably render the conjunctiva susceptible to infection.

These studies suggest the importance of investigating the paraocular glands in other types of conjunctival infection. It is possible that they may have a definite relation to the origin and course of such infections.

We wish to express our indebtedness to Miss Eunice E. Chace, of the Department of Zoology of Smith College, for references and other data respecting the anatomy of the lacrimal and Harderian glands.

EXPLANATION OF PLATES.

PLATE 7.

FIG. 1, a and *b*. (*a*) Lacrimal gland. (*b*) Harderian gland, showing the close relation of the two structures and the normal histology of each. Many of the acini of the lacrimal gland show no lumen, whereas the lumina of the acini of the Harderian gland are comparatively large. × 100.

FIG. 2. Harderian gland showing comparatively normal acini. The free margins of the cells are ragged. The lumina contain yellow, granular precipitate. Zenker fixation.

PLATE 8.

FIG. 3. Harderian gland showing parenchymatous changes. The cytoplasm of the cells is ragged and contains numerous vacuoles.

FIG. 4. Rat with advanced ophthalmia. Necrosis of epithelium involving one lobule of the Harderian gland.

PLATE 9.

FIG. 5. Rat with well marked ophthalmia. General dilatation of the acini of the Harderian gland. × 100.

FIG. 6. Suppurative inflammation of the Harderian gland. The tubules are filled with disintegrating polymorphonuclear leucocytes.

PLATE 10.

FIG. 7. Old rat with chronic conjunctivitis. Diffuse chronic inflammatory process in the Harderian gland with dilatation of the ducts, atrophy of the acini, marked increase in connective tissue, and widespread mononuclear cell infiltration. × 100.

FIG. 8. Complete atrophy of a lobule of the Harderian gland with replacement by fat and connective tissue. × 100.

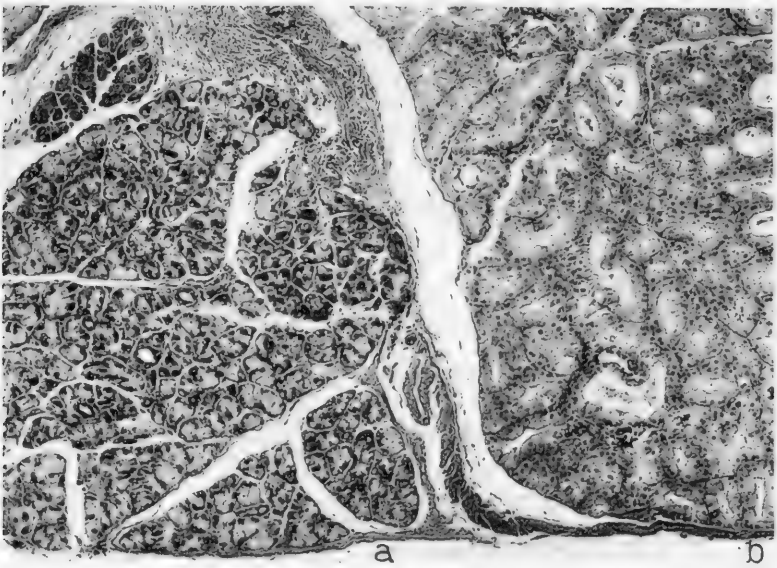

FIG. 1.

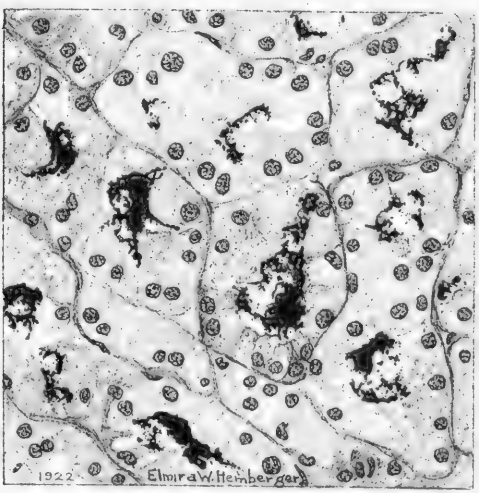

FIG. 2.

(Lambert and Yudkin: Deficiency of vitamine A.)

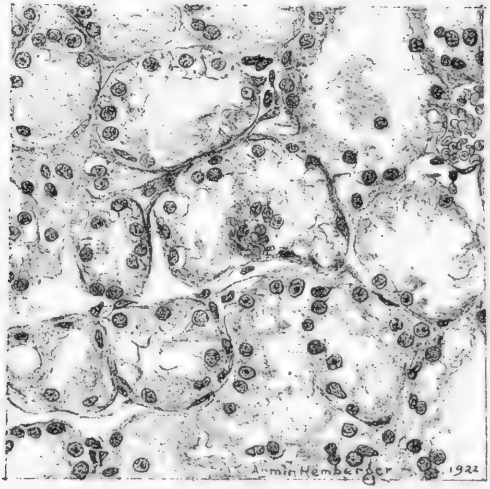

FIG. 3.

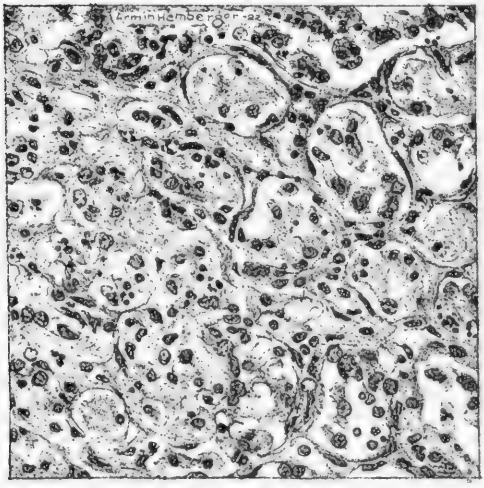

FIG. 4.

(Lambert and Yudkin: Deficiency of vitamine A.)

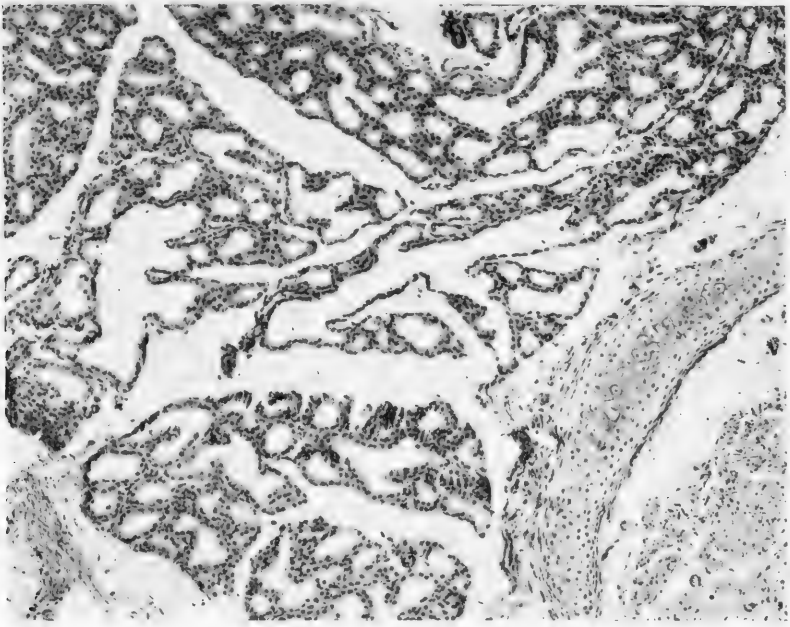

FIG. 5.

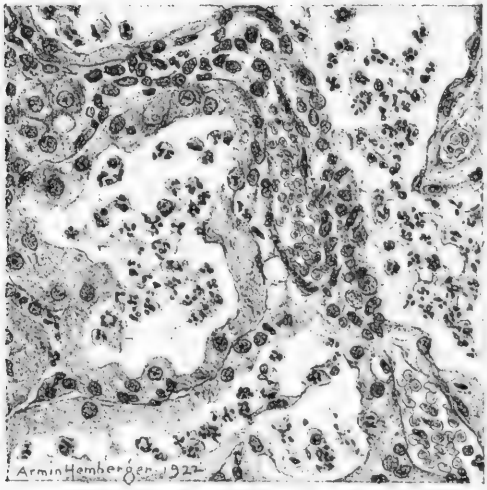

FIG. 6.

(Lambert and Yudkin: Deficiency of vitamine A.)

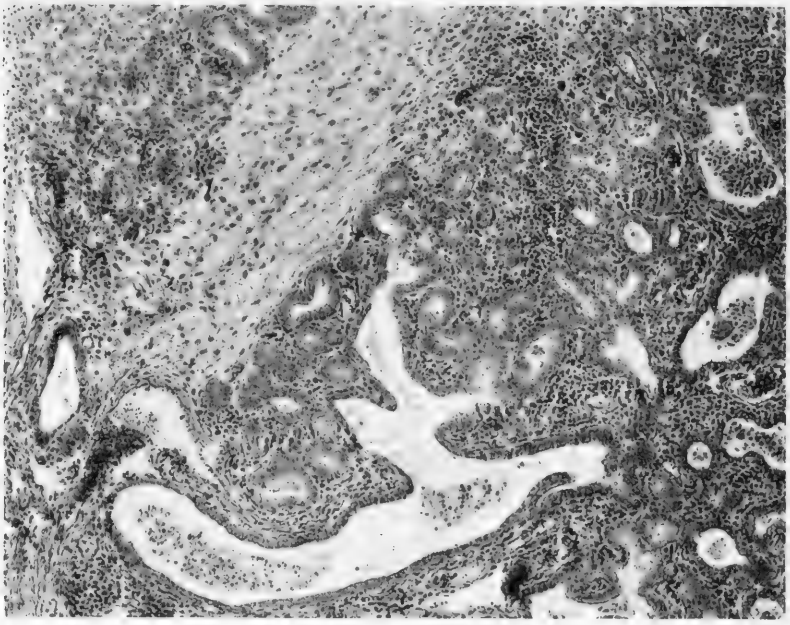

FIG. 7.

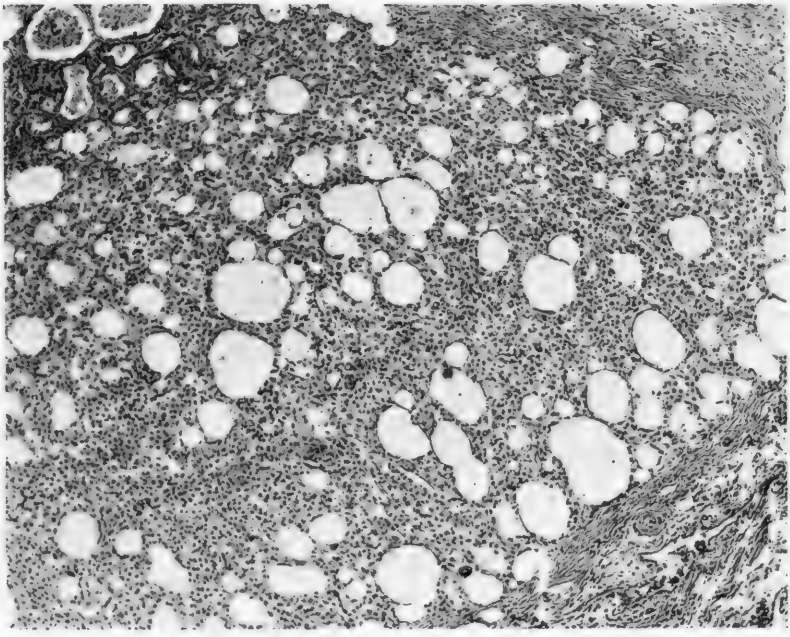

FIG. 8.

(Lambert and Yudkin: Deficiency of vitamine A.)

MICROBIC VIRULENCE AND HOST SUSCEPTIBILITY IN PARATYPHOID-ENTERITIDIS INFECTION OF WHITE MICE. I.

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Earlier reports [1–3] on the study of mouse typhoid infection have been concerned with a single strain of bacilli. We wish now to describe a series of experiments on mice with six related strains of paratyphoid-enteritidis bacilli isolated from man and animals. In a first communication we propose to consider the effect of repeated intraperitoneal passage on the virulence of the several strains and in a second[4] to analyze the equilibrium between the virulence of each strain and the individual and group resistances of the various mice employed.

EXPERIMENTAL.

The mice were raised at The Rockefeller Institute in the breeding station already described.[2] Animals of one age and nearly uniform weight were chosen and fed on a diet of bread and milk. For reasons of economy of space and care two mice were placed in each 8 by 10 inch battery jar. The strains of paratyphoid-enteritidis bacilli used for inoculation have been described in earlier papers[2,3] and can be identified from their designations which follow: Mouse Typhoid I (M. T. I), *Bacillus enteritidis* (Gaertner), Mouse Typhoid II (M. T.

[1] Flexner, S., *J. Exp. Med.*, 1922, xxxvi, 9.

[2] Amoss, H. L., *J. Exp. Med.*, 1922, xxxvi, 25, 45. Amoss, H. L., and Haselbauer, P. P., *J. Exp. Med.*, 1922, xxxvi, 107.

[3] Webster, L. T., *J. Exp. Med.*, 1922, xxxvi, 71, 97; 1923, xxxvii, 21, 33, 231, 269.

[4] Webster, L. T., *J. Exp. Med.*, 1923, xxxviii, 45.

II), *Bacillus pestis caviæ*, *Bacillus aertrycke* (mutton), and *Bacillus paratyphosus* B.[5]

In dealing with these several strains in which the cultural and serological reactions are so nearly alike, particular care is required in order to avoid error. To emphasize this point, we have interpolated a table which summarizes the fermentation and agglutination reactions of the several strains and indicates their relative pathogenicity for mice (Table I).

Experiment 1.—The six strains grown on agar slants were transferred to broth, incubated for 18 hours, and then diluted as follows: 1:10, 1:100, 1:1,000, 1:10,000, 1:100,000, and 1:1,000,000. Two mice were given intraperitoneally 1 cc. of each dilution. By the usual plating method, the number of bacteria per cubic centimeter in each dilution was estimated. All the mice succumbing were submitted to autopsy and cultures made from the heart's blood and spleen. The growths obtained were checked by tests outlined in Table I.

The identified broth culture from the heart's blood of the first mouse to die of each series after suitable dilutions was used to inject the next series of mice. Each of the six strains underwent from four to eight passages in this manner; control titrations were then made with the original unpassed strains taken directly from the stock agar tube.

Tables II and III illustrate the manner of recording duration of life of the mice used in each passage of every strain. Text-figs. 1 to 6 plot the results of the entire experiment in a convenient manner by showing the virulence of the given strain in five different dilutions for each consecutive passage. No progressive increase in virulence for each passage in any dilution of any strain or appreciable difference between the virulence of the passed culture and the control unpassed culture was observed. The occasional discrepancy in the otherwise uniform results will be accounted for later on the basis of differences in individual host susceptibility.

Text-fig. 7 compares the intraperitoneal virulences of the six strains in four different dilutions. It may be seen that in all dilutions,

[5] M. T. I is a mouse typhoid strain isolated from a spontaneous case and closely related culturally and serologically to *B. enteritidis* (Gaertner). M. T. II is the mouse typhoid strain employed throughout our series of studies. It has been identified with *B. pestis caviæ* and related very closely to *B. aertrycke* (mutton).

B. pestis caviæ, *B. enteritidis* (Gaertner), *B. aertrycke* (mutton), and *B. paratyphosus* B are stock strains obtained from Dr. Charles Krumwiede.

TABLE I.

Differentiation of Six Paratyphoid-Enteritidis Strains.

Strain.	Cultural reactions.								Agglutination titer in immune sera.					Pathogenicity for mice.
	Glucose.	Maltose.	Xylose.	Arabinose.	Lactose.	Saccharose.	Lead acetate reduction.	Inosite.	M. T. I.	M. T. II.	*B. pestis cavie.*	*B. aertrycke* (mutton).	*B. paratyphosus* B.	
M. T. I.	+	+	+	+	−	−	+	−	1:10,000 ++	−	−	−	−	High.
B. enteritidis	+	+	+	+	−	−	+	−	+	−	−	−	−	"
M. T. II.	+	+	+	+	−	−	+	+	−	1:10,000 ++	+1	+1	+	Moderate.
B. pestis caviæ	+	+	+	+	−	−	+	−	−	+	1:3,000 ++	+	1 or −	Low.
" *aertrycke* (mutton)	+	+	+	+	−	−	+	−	−	+	+1	1:4,000 ++	+	Moderate.
" *paratyphosus* B	+	+	+	+	−	−	+	+	−	− or 1	−	−	1:3,000 ++	Low.

Agglutination test symbols: ++, complete agglutination to titer; +1, agglutination, cloudy supernatant fluid, to titer; +, fair agglutination to titer; 1, trace of agglutination to titer; −, no agglutination at titer.

TABLE II.

Intraperitoneal Passage of Strain M. T. I.

Passage No.	No. of bacteria per mouse.	No. of mice.	Duration of life.	No. of bacteria per mouse.	No. of mice.	Duration of life.	No. of bacteria per mouse.	No. of mice.	Duration of life.	No. of bacteria per mouse.	No. of mice.	Duration of life.	No. of bacteria per mouse.	No. of mice.	Duration of life.	No. of bacteria per mouse.	No. of mice.	Duration of life.
			days			*days*			*days*			*days*			*days*			*days*
2	19,000,000,000	2	1, 1	1,900,000,000	2	1, 1	190,000,000	2	1, 1	19,000,000	2	1, 20*	1,900,000	2	1, 3	190,000	2	1, 1
3	1,500,000,000	2	1, 1	150,000,000	2	1, 2	15,000,000	2	1, 2	1,500,000	2	1, 1	150,000	2	1, 2	15,000	2	4, 6
4	500,000,000	2	1, 1	50,000,000	2	1, 1	5,000,000	2	1, 1	500,000	2	1, 1	50,000	2	2, 3	5,000	2	2, 6
5	5,000,000	2	1, 1	500,000	2	1, 2	50,000	2	4, 4	5,000	2	2, 4	500	2	S., S.†			
6	4,000,000	2	1, 1	400,000	2	1, 2	40,000	2	3, S.	4,000	2	5, 6	400	2	11, 11			
7	4,000,000	2	1, 2	400,000	2	2, 2	40,000	2	2, 4	4,000	2	4, 4	400	2	4, 5			
8	3,000,000	2	1, 1	300,000	2	2, 3	30,000	2	2, 2	3,000	2	3, 3	300	2	5, 11			
C.	2,500,000	2	1, 2	250,000	2	2, 2	25,000	2	3, 6	2,500	2	2, 12	250	2	2, 8			

* Autopsy cultures negative.

† S. indicates that the animal survived for 30 days; C. indicates control.

TABLE III.

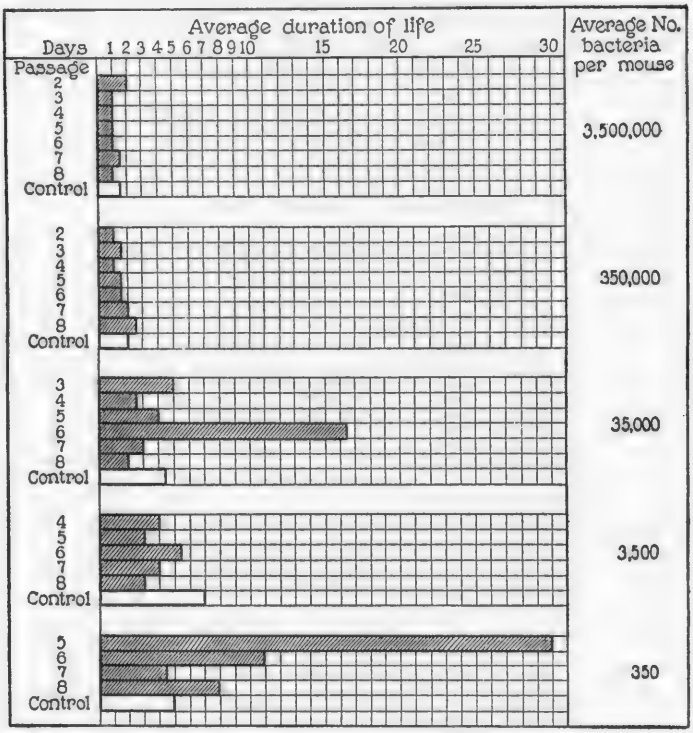
Intraperitoneal Passage of Strain B. pestis caviæ.

Passage No.	No. of bacteria per mouse.	No. of mice.	Duration of life.	No. of bacteria per mouse.	No. of mice.	Duration of life.	No. of bacteria per mouse.	No. of mice.	Duration of life.	No. of bacteria per mouse.	No. of mice.	Duration of life.	No. of bacteria per mouse.	No. of mice.	Duration of life.	No. of bacteria per mouse.	No. of mice.	Duration of life.
			days			*days*			*days*			*days*			*days*			*days*
2	19,000,000,000	2	1, 1	1,900,000,000	2	1, 1	190,000,000	2	1, 1	19,000,000	2	1, 4	1,900,000	2	4, 7	190,000	2	6, 9
3	250,000,000	2	1, 1	25,000,000	2	1, 1	2,500,000	2	1, 1	250,000	2	4, 6	25,000	2	6, S.	2,500	2	8, 27*
4	200,000,000	2	1, 1	20,000,000	2	1, 1	2,000,000	2	1, 2	200,000	2	3, 4	20,000	2	5, 6	2,000	2	6, 10
5	3,500,000	2	1, 2	350,000	2	3, 4	35,000	2	5, 6	3,500	2	S., S.†	350	2	S., S.			
6	2,500,000	2	2, 5	250,000	2	3, 5	25,000	2	2, 3	2,500	2	8, 12	250	2	" "			
7	3,000,000	2	2, 5	300,000	2	4, 4	30,000	2	4, 4	3,000	2	10, 11	300	2	" "			
8	2,000,000	2	3, 5	200,000	2	5, 13	20,000	2	5, 8	2,000	2	S., S.	200	2	" "			
C.	2,000,000	2	3, 3	200,000	2	1, 3	20,000	2	5, 6	2,000	2	8, "	200	2	25, "			

* Autopsy cultures negative.

† S. indicates that the animal survived for 30 days; C. indicates control.

Strains M. T. I and *Bacillus enteritidis* are most virulent and are quite similar; that Strains M. T. II and *Bacillus aertrycke* (mutton) have the same degree of virulence, which is less than the enteritidis

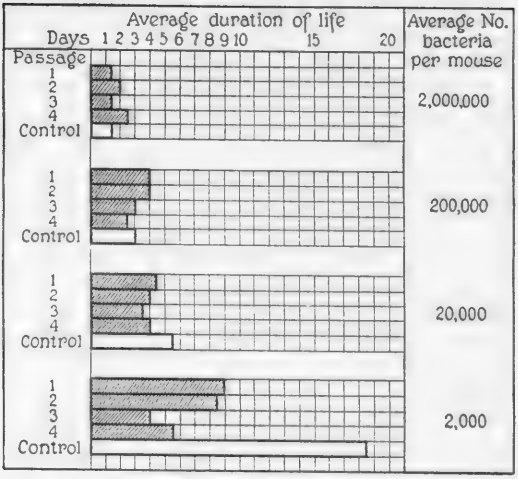


TEXT-FIG. 1. Intraperitoneal virulence of Strain M. T. I.

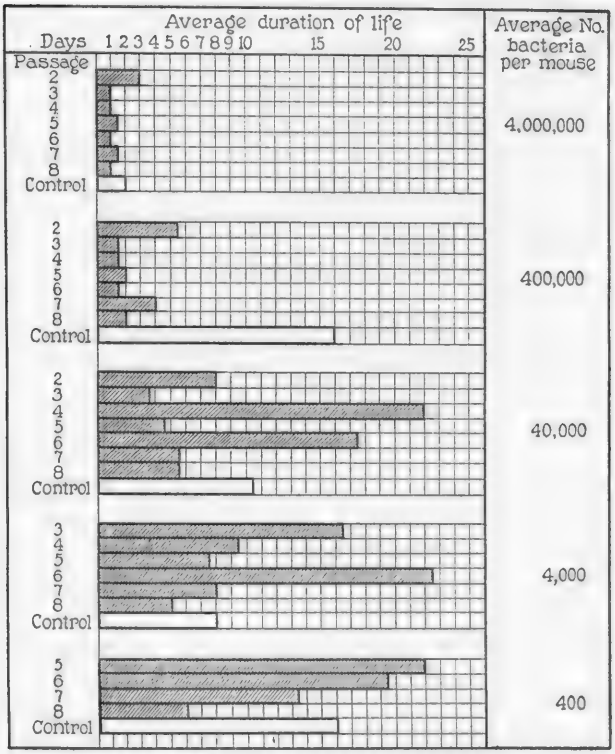
In Text-figs. 1 to 6 the "Average duration of life in days" represents the mean survival time of the two mice used for each dilution. "Average number of bacteria per mouse" represents the mean of the nearest comparable figures for each passage.

strains; and that *Bacillus pestis caviæ* and *Bacillus paratyphosus* B show the least virulence.

In brief, then, under the conditions of the experiment, intraperitoneal mouse passage did not affect the virulence of six strains of paratyphoid-enteritidis bacilli.

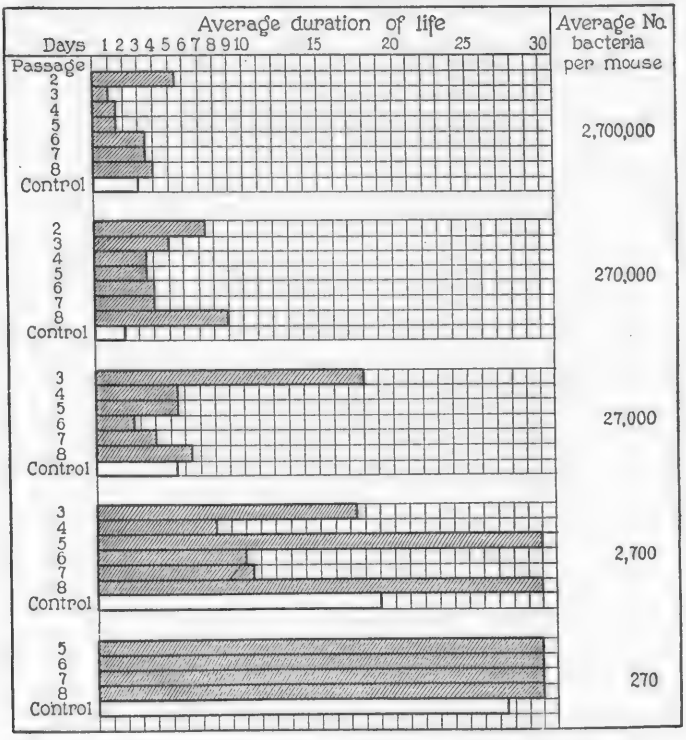


TEXT-FIG. 2. Intraperitoneal virulence of Strain *B. enteritidis*.



TEXT-FIG. 3. Intraperitoneal virulence of Strain M. T. II.

Experiment 2 was planned to determine the effect of direct intraperitoneal mouse passage without intermediate artificial cultivation on the virulence of the six strains of paratyphoid-enteritidis bacilli.

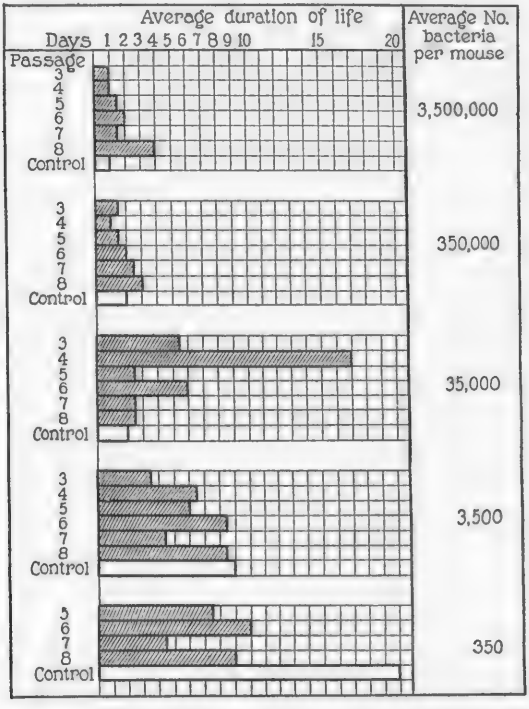


TEXT-FIG. 4. Intraperitoneal virulence of Strain *B. pestis caviæ*.

Method.—Broth cultures from the six stock agar tubes were incubated 6 hours and injected in 1 cc. quantities each intraperitoneally into three mice. 24 hours later, these mice were either dead or dying. From the living mice, 0.5 cc. of blood was taken from the heart and mixed with an equal volume of 2 per cent sodium citrate in 0.85 per cent salt solution. The heart and spleen from the dead mice of the same series were ground in citrate solution in a mortar with sand and combined with the blood suspension.[6] The six resulting emulsions were shaken and diluted

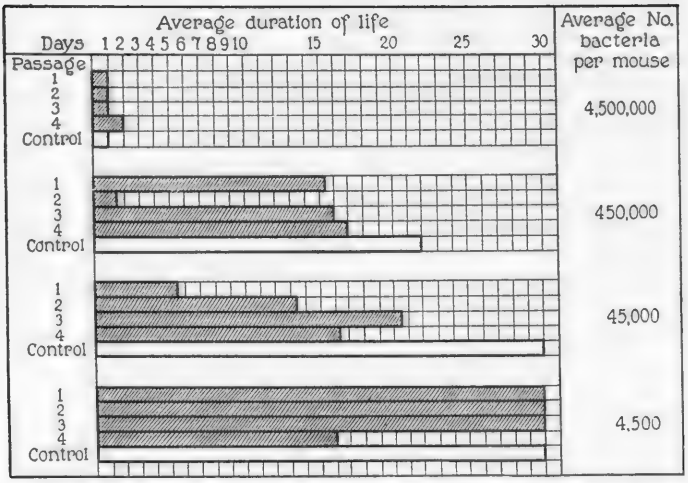
[6] Preliminary test showed that the bacteria taken from the blood during the course of a rapid fatal septicemia do not differ in virulence from those recovered 1 or 2 hours after death from spleen and heart emulsions.

1:100, so that each contained about 3,000,000 organisms per cc. 1 cc. of this dilution of the emulsions was injected intraperitoneally into two mice. 24 hours later, these mice were found in a dying condition. The above procedure was then repeated and continued for five passages. The sixth or final passages were made as follows: dilution 1:10, two mice; 1:100, five mice; 1:1,000, five mice; and 1:10,000, two mice. At this time, control inoculations were made with 18 hour broth cultures from the stock agar tubes in the following dilutions: 1:100, two mice; 1:1,000, five mice; 1:10,000, five mice; and 1:100,000, two mice. The number of colonies per cubic centimeter of each culture was determined by dilution methods so that actual numbers of bacteria per mouse could be used in computing degrees of virulence.

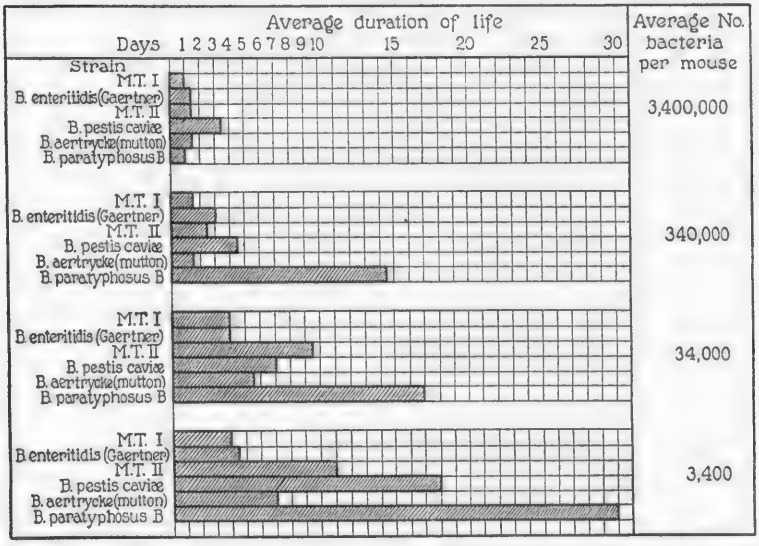


TEXT-FIG. 5. Intraperitoneal virulence of Strain *B. aertrycke* (mutton).

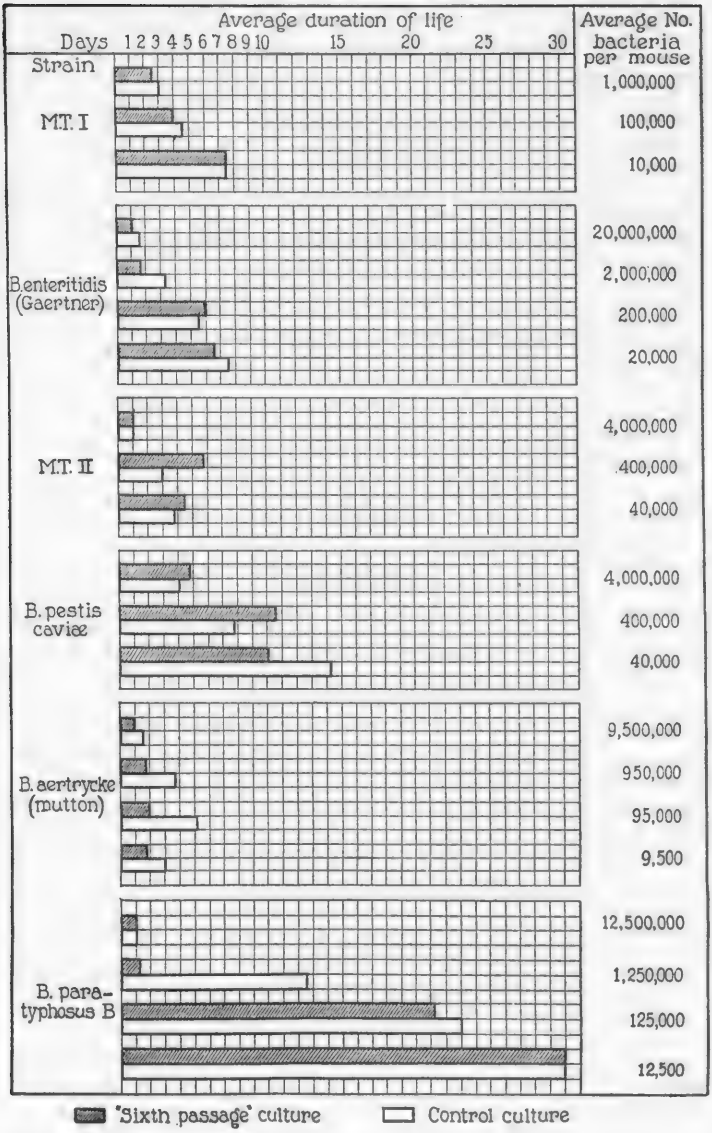
Text-fig. 8, which plots the results of this experiment in the manner described for Text-figs. 1 to 7, shows that direct intraperitoneal mouse passage without intermediate artificial cultivation does not affect the virulence of Strains M. T. I, *Bacillus enteritidis*, M. T. II, and *Bacillus pestis caviæ*. However, there does seem to be a slight



TEXT-FIG. 6. Intraperitoneal virulence of Strain *B. paratyphosus* B.



TEXT-FIG. 7. Intraperitoneal virulences of six paratyphoid-enteritidis strains. "Average duration of life in days" represents the mean survival time of all animals in all passages of the particular strain receiving roughly the same number of bacteria. "Average number of bacteria per mouse" represents the mean of the nearest comparable figures for each of the six series.



TEXT-FIG. 8. Effect of direct passage on the intraperitoneal virulences of six paratyphoid-enteritidis strains.

increase in virulence for each dilution of *Bacillus aertrycke* (mutton) and in one dilution for *Bacillus paratyphosus* B, but when the average duration of life of the passed culture is compared in the various dilutions with the average results shown in Text-fig. 7, the small differences actually become negligible.

From these two experiments we may conclude that direct intraperitoneal passage from mouse to mouse of certain strains of paratyphoid-enteritidis bacilli does not affect materially the basic virulence.

A discussion of the above findings and a further summary will be given in connection with the next paper of the series.[4]

MICROBIC VIRULENCE AND HOST SUSCEPTIBILITY IN PARATYPHOID-ENTERITIDIS INFECTION OF WHITE MICE. II.

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(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

(Received for publication, March 15, 1923.)

Experimental studies[1] indicate that strains of paratyphoid-enteritidis bacilli differ among themselves in virulence but that for each strain, virulence is relatively a fixed property. It seemed desirable, therefore, to test this point by a series of *per os* passages which would at the same time afford an opportunity to observe infection under more usual conditions and to analyze more accurately the relations existing between the host and the microbic invader.

EXPERIMENTAL.

Experiment 1.—The method adopted has been described elsewhere.[2] Mice weighing 16 to 18 gm. each, taken from the stock breeding room, were segregated two per jar. The six cultures used were obtained from the last intraperitoneal passage reported in the previous paper.[1] Twenty mice were used for each passage of every strain. The dose, 0.5 cc. *per os* of a 16 hour broth culture diluted 1:100, contained approximately 3,000,000 bacteria. The dead mice were autopsied and the cultures obtained from them were identified. From the first mouse to die of each series a blood culture from the heart was made, identified, and after 24 hours incubation, injected into another series of twenty mice. This procedure was repeated with each strain for a varying number of passages and was followed by a control passage with the original, unpassed stock culture. Then it was considered desirable to return to the cultures used for the first *per os* passages and conduct a duplicate series of passages, terminated as before, by a control passage with the original stock culture. Blood cultures were made on the last control series. The technique of this procedure has been described in a previous paper.[2]

Tables I to IV, which illustrate the methods of recording data in each series, show the duration of life and results of blood cultures

[1] Webster, L. T., *J. Exp. Med.*, 1923, xxxviii, 33.

[2] Webster, L. T., *J. Exp. Med.*, 1923, xxxvii, 231.

following injection with *Bacillus enteritidis* and *Bacillus paratyphosus* B respectively. In Text-figs. 1 to 5 are plotted typical curves from each series and in Text-fig. 6 the curves for each strain are compared with one another and with the control Strain M. T. II.

TABLE I.

Per Os Passage. Strain B. enteritidis.

Passage No.	No. of bacteria per mouse.	No. of mice.	Duration of life after injection.	No. of survivors after 60 days.
			days	
1	4,000,000	20	5, 7, 8, 8, 9, 9, 9, 11, 11, 11, 11, 11, 11, 14, 16, 20*, 21, 39*	2
2	3,100,000	20	6, 8, 8, 9, 9, 9, 10, 12, 12, 12, 14, 15, 15, 18, 21, 22, 56*	3
3	3,400,000	20	5, 8, 8, 9, 11, 12, 13, 14, 19, 20, 21, 25, 26, 38, 40, 53, 55	3
4	3,000,000	18	5, 6, 7, 7, 8, 8, 8, 8, 9, 9, 9, 9, 9, 9, 10, 12, 12, 18	0
Control.	3,000,000	20	6, 6, 8, 8, 10, 10, 10, 10, 10, 11, 11, 12, 12, 13, 14, 15, 26	3
1 A	3,500,000	20	6, 8, 8, 8, 8, 8, 9, 9, 9, 9, 9, 10, 10, 10, 12, 15, 16, 28	2
2 A	4,000,000,	20	6, 6, 7, 7, 7, 8, 8, 8, 9, 11, 11, 11, 11, 11, 14, 26, 36	3
Control 2.	5,000,000	20	7, 8, 8, 8, 8, 8, 8, 8, 9, 10, 10, 10, 10, 11, 11, 11, 12, 12, 16, 29	0

* Autopsy cultures sterile.

TABLE II.

Per Os Passage. Strain B. paratyphosus B.

Passage No.	No. of bacteria per mouse.	No. of mice.	Duration of life after injection.	No. of survivors after 60 days.
			days	
1	4,000,000	19	18*, 19, 20*, 21†	15
1 A	5,000,000	20	13, 14†, 14†, 20, 22, 29†, 30	13
2 A	5,000,000	18	8, 14†, 16, 28	16
3 A	3,000,000	20	13, 21	18
Control.	3,000,000	20	6, 18, 19†, 25*	16

* Autopsy cultures sterile.

† Autopsy cultures not identified.

TABLE III.

Blood Cultures from Control 2 Series Mice Inoculated per Os with B. enteritidis on January 31, 1923.

Cage and mouse.	Cultured Feb. 1.	Cultured Feb. 3.	Cultured Feb. 5.	Cultured Feb. 7.	Cultured Feb. 9.	Cultured Feb. 14.	Cultured Feb. 21.	
1 A	−	1 col.	++	+++	Feb. 7. D.			
B	−	−	−	+	1 col.	++	Feb. 15. D.	
2 A	−	−	+++	Feb. 7. D.				
B	−	−	+++	" 7. "				
3 A	−	−	−	+	+++	Feb. 10. D.		
B	−	−	+++	++	Feb. 9. D.			
4 A	−	−	++	++	" 9. "			
B	−	−	+	+++	" 8. "			
5 A	−	−	++	Feb. 7. D.				
B	−	−	+	+	+++	Feb. 10. D.		
6 A	−	−	+++	Feb. 7. D.				
B	−	++	+++	" 6. "				
7 A	−	−	+	−	Feb. 9. D.			
B	−	−	+	−	++	Feb. 11. D.		
8 A	−	−	+++	Feb. 7. D.				
B	−	−	++	++	Feb. 9. D.			
9 A	−	−	+	+	1 col.	−	++	Feb. 28. D.
B	−	−	+++	Feb. 7. D.				
10 A	−	−	+	++	+++	Feb. 10. D.		
B	−	−	+	++	+++	" 11. "		

+ indicates 1 to 10 colonies; ++, 10 to 50 colonies; and +++, more than 50 colonies.

The tables and curves indicate that repeated *per os* passage does not alter the virulence of these strains. Strains M. T. I and *Bacillus enteritidis* are shown to have been most virulent and to act similarly.

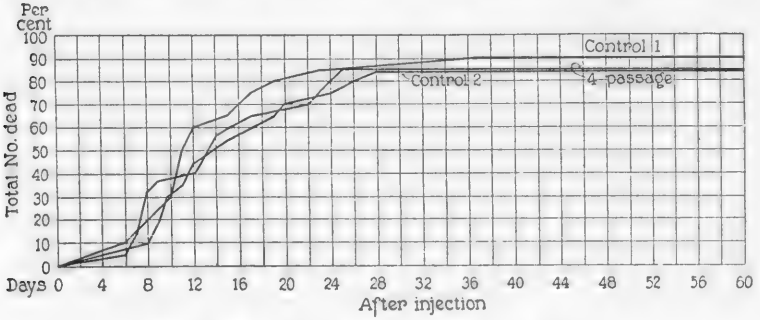
TABLE IV.

Blood Cultures from Control 1 Series Mice Inoculated per Os with B. paratyphosus B on February 8, 1923.

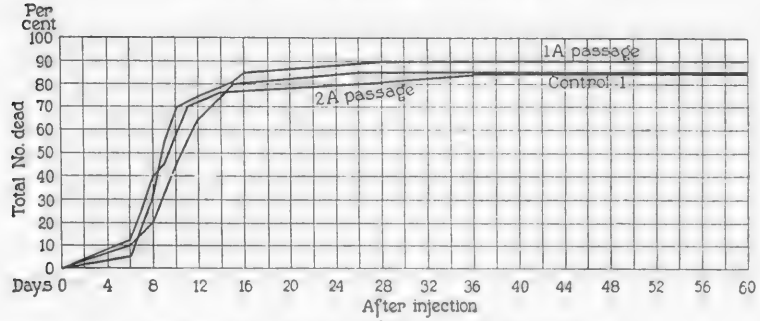
Cage and mouse.	Cultured Feb. 9.	Cultured Feb. 14.	Cultured Feb. 16.	Cultured Feb. 21.	Cultured Feb. 27.	Cultured Mar. 1.	Cultured Mar. 7.	Cultured Mar. 14.	Cultured Mar. 21.
1 A	–	–	–	–	–	–	–	–	–
B	–	–	–	–	–	–	–	–	–
2 A	–	–	–	–	–	–	–	–	–
B	–	–	–	–	–	–	–	–	–
3 A	–	–	–	–	–	–	–	–	–
B	–	–	–	–	–	–	–	–	–
4 A	–	–	–	–	–	–	–	–	–
B	–	–	–	–	–	–	–	–	–
5 A	–	–	–	–	Feb. 26. D.				
B	–	–	–	–	–	–	–	–	–
6 A	–	–	–	–	–	–	–	–	–
B	–	–	–	–	–	–	–	–	–
								1 col. M. T. I.	
7 A	–	–	–	–	–	–	Mar. 5. D.		
B	–	Feb. 14. D.							
8 A	–	+++	–	–	Feb. 27. Killed by accident.				
B	++	+++	–	1 col.	–	–	–	–	–
9 A	–	–	–	–	–	–	–	–	–
B	–	–	–	–	–	–	–	–	–
10 A	–	–	–	–	–	–	–	–	–
B	–	–	–	–	–	–	–	–	–

+ indicates 1 to 10 colonies; ++, 10 to 50 colonies; and +++, more than 50 colonies.

After *per os* injection positive blood cultures developed promptly and 80 to 90 per cent of the mice died within 3 weeks. Strains M. T. II and *Bacillus aertrycke* (mutton) proved to be less virulent and behaved quite alike. About the same percentage of mice survived, some having shown persistently negative blood cultures, and a few having shown positive cultures and agglutinins. Strains *Bacillus*



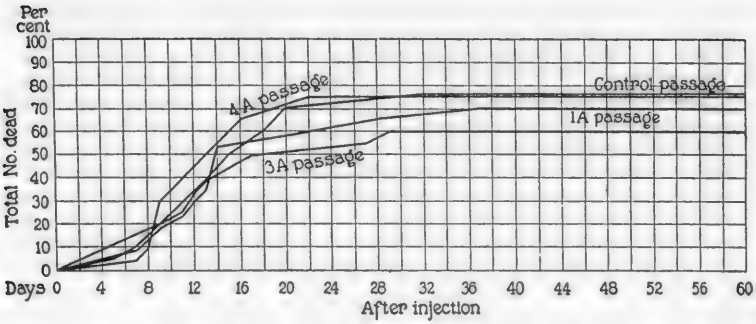
TEXT-FIG. 1. *Per os* passage of Strain M. T. I.



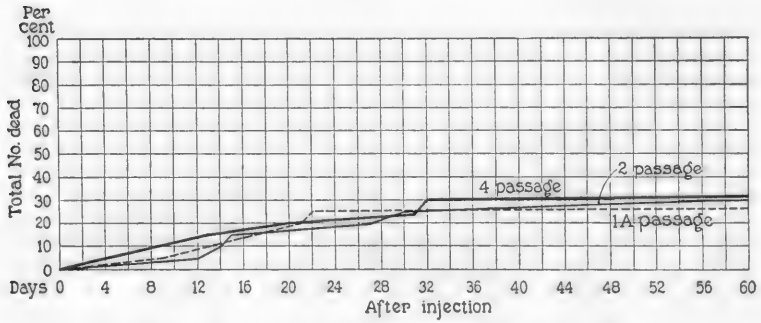
TEXT-FIG. 2. *Per os* passage of Strain *B. enteritidis*.

pestis caviæ and *Bacillus paratyphosus* B affected very few mice in any passage and usually did not invade the blood stream.

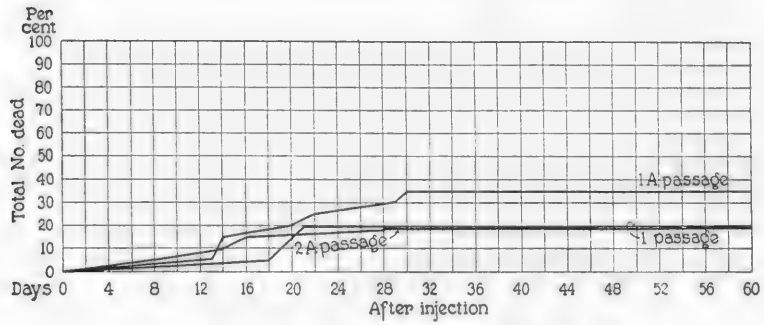
The fixed individual differences in susceptibility to the several pathogenic strains observed among the mice indicated that the reaction was of non-specific nature. In order to test this possibility a simple poisonous chemical compound was administered to a group of mice.



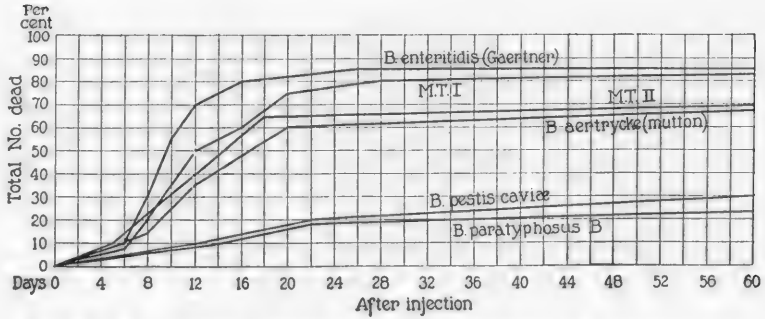
TEXT-FIG. 3. *Per os* passage of Strain *B. aertrycke* (mutton).



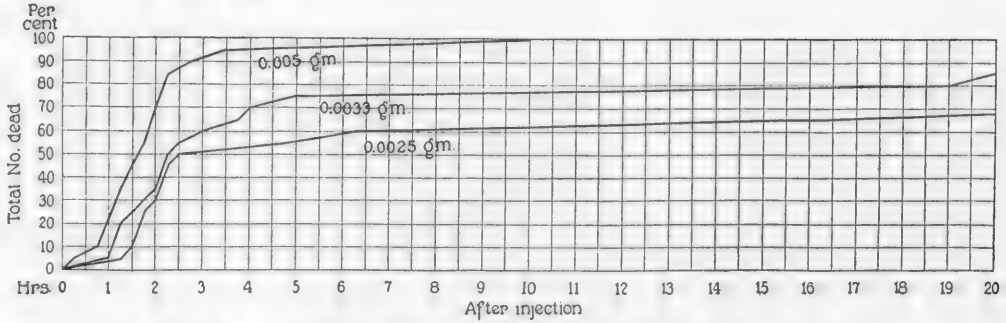
TEXT-FIG. 4. *Per os* passage of Strain *B. pestis caviæ.*



TEXT-FIG. 5. *Per os* passage of Strain *B. paratyphosus* B.



TEXT-FIG. 6. Standard mortality curve of six strains of the paratyphoid-enteritidis group.



TEXT-FIG. 7. Mortality curve following *per os* injections of mercury bichloride.

Experiment 2.—From a flask containing 1 gm. of mercury bichloride dissolved in 100 cc. of hot distilled water, subsequent dilutions were made in the usual bacteriological manner more to parallel biological technique than to attain chemical accuracy. The various doses in 0.5 cc. volume were administered *per os* by stomach tube to mice weighing about 16 gm. each.

Test 1: Two mice received 0.05 gm. each, two 0.005 gm., two 0.0005 gm., and two 0.00005 gm. The first two mice were dead in 5 minutes; one receiving 0.005 gm. died in 60 minutes, the other in 18 hours. The remaining mice survived 30 days.

Test 2: Two mice received 0.005 gm. each, two 0.0025 gm., two 0.00125 gm., two 0.0008 gm., and two 0.00061 gm. The 0.005 gm. mice died in 2 hours; the 0.0025 gm. mice were dead in 36 hours; the 0.00125 gm. mice were dead in 72 hours; the remainder survived 30 days.

Test 3: Twenty mice received 0.005 gm. each, twenty 0.0033 gm., and twenty 0.0025 gm. Text-fig. 7 shows the duration of life of these three series.

The mortality curve in Text-fig. 7, following 0.0033 gm. *per os* injection, is similar in form to that resulting from *per os* injection of Strain M. T. I or *Bacillus enteritidis* (Text-figs. 1 and 2), and the curve following the 0.0025 gm. injection resembles the M. T. II and *Bacillus aertrycke* (mutton) curves (Text-fig. 6). The comparison in the latter instance is of special interest since it has been shown previously[3] that the M. T. II curve (Text-fig. 6) may be superimposed upon the Amoss experimental epidemic curves.

Hence it appears that by an adjustment of the dose of mercury bichloride it is possible to secure mortality curves agreeing in form with those resulting from the *per os* injection of living bacteria.

DISCUSSION.

We wish now to correlate our findings with the results of earlier investigations along similar lines. Loeffler[4] and Danysz[6] made the first systematic studies on this group of bacilli in mice, rats, and guinea pigs. While it is true that their main purpose may be regarded as the perfecting of a method for the extermination of rodents, yet they and their successors incidentally or otherwise dealt with such

[3] Webster, L. T., *J. Exp. Med.*, 1923, xxxvii, 269.
[4] Loeffler, F., *Centr. Bakt.*, 1892, xi, 129; xii, 1.
[5] Mereshkowsky, S. S., *Centr. Bakt., 1te Abt.*, 1895, xvii, 742.
[6] Danysz, J., *Ann. Inst. Pasteur*, 1900, xiv, 193.

questions as the natural spread of the infection in nature and the concurrent fluctuations of virulence among the bacilli.[4–20]

Perhaps it was Danysz who first emphasized the variations of the virulence in order to explain the rise and fall in mortality among the rodents exposed. He was followed by a number of investigators more or less upholding his views on this question, and lately Topley has also adopted this hypothesis as a partial explanation for the epidemic curves obtained in his studies of mouse typhoid.[21] There is, however, no real agreement in the opinions expressed, which range from statements that the virulence fluctuates[6,10,14,15,20] to statements that it remains constant,[13] or that the experimental results are irregular and not interpretable.[8,12,16,17]

We have been impressed with the lack of accuracy in respect to the constancy of dosage, and the small number of animals employed for the experimental tests. Hence our plan included using larger numbers of mice amounting in the end to about 1,400, and controlling the dosage of the bacilli. From the results, which in our case have been generally consistent, we believe that the following may be postulated:

First, the virulence of each of several strains of paratyphoid-enteritidis bacilli is apparently of fixed quantity, for mice at least. It would seem that since the strains used in the present experiments were originally isolated from man and several other animal species

[7] Klein, E., and Williams, H., *Lancet*, 1901, ii, 440.

[8] Kister, J., and Köttgen, P., *Deutsch. med. Woch.*, 1901, xxvii, 275.

[9] Krausz, A., *Deutsch. med. Woch.*, 1901, xxvii, 351.

[10] Bronstein, J., *Deutsch. med. Woch.*, 1901, xxvii, 577.

[11] Abel, R., *Deutsch. med. Woch.*, 1901, xxvii, 869.

[12] Rosenau, M. J., *Bull. Hyg. Lab., U.S.P.H., No. 5*, 1901.

[13] Issatschenko, B., *Centr. Bakt., 1te Abt., Orig.*, 1902, xxxi, 26.

[14] Markl, G., *Centr. Bakt., 1te Abt., Orig.*, 1902, xxxi, 202.

[15] Bahr, L., *Centr. Bakt., 1te Abt., Orig.*, 1905, xxxix, 263.

[16] Trautmann, H., *Z. Hyg. u. Infectionskrankh.*, 1906, liv, 104.

[17] Xylander, *Arb. k. Gsndhtsamte*, 1908, xxviii, 145.

[18] Bainbridge, F. A., *J. Path. and Bact.*, 1909, xiii, 443.

[19] Schern, K., *Arb. k. Gsndhtsamte*, 1909, xxx, 575.

[20] Steffenhagen, K., *Arb. k. Gsndhtsamte*, 1911, xxxvi, 198.

[21] Topley, W. W. C., *Lancet*, 1919, ii, 1; *J. Hyg.*, 1920–21, xix, 350; 1921, xx, 103. Topley, W. W. C., Weir, H. B., and Wilson, G. S., *J. Hyg.*, 1921, xx, 227. Topley, W. W. C., *J. Hyg.*, 1922, xxi, 10, 20.

and cultivated artificially for longer or shorter periods of time, there is good reason for supposing that the virulence of each for mammals in general is relatively a constant. It therefore seems permissible to exclude the factor of fluctuating virulence of the bacilli as explaining the epidemic mortality curve of mouse typhoid.

Next, it is clear from our studies that the different strains of paratyphoid-enteritidis bacilli vary among themselves in virulence for mice. Doubtless similar variations occur for other animals, including man. This inherent pathogenicity becomes, then, an important factor which may determine a severe outbreak of infection or the reverse among a susceptible group of animals. As the strains of high and low virulence may be antigenically identical, the transformation of one into the other cannot be predicated on the basis of bacteriological findings in sick or dead animals. In other words, to determine such transition, it is necessary to employ only accurately planned experiments in which the danger of substitution has been eliminated.

Finally, the experiments have brought out the fact, interesting and perhaps fundamental, that the degree of individual host susceptibility as exhibited by the mortality curve is a general property not restricted to the bacterial infections which we have been studying but inclusive of a chemical intoxication as, for example, with mercury bichloride. It is of importance to know that even when as few as twenty individual mice of a given age and weight, reared alike, are assembled, so small a group will contain individuals highly subject to infection or intoxication, as the case may be, individuals very resistant to the same injurious agencies, and individuals standing between these extremes.

CONCLUSIONS.

1. Six strains of paratyphoid-enteritidis bacilli, isolated from man and animals, differed markedly in virulence for mice.

2. The inherent virulence of each strain for mice was not affected by repeated intraperitoneal and *per os* mouse passage.

3. Individual differences in the susceptibility of the mice to each strain and to graded doses of mercury bichloride were demonstrable. Such variations appear to be of a non-specific nature.

We wish to thank Miss Allen Johnson for assisting with the technical work involved in the experiments reported in this and the preceding paper of the series.[1]

THE EFFECT OF SODIUM CHLORIDE ON THE CHEMICAL CHANGES IN THE BLOOD OF THE DOG AFTER PYLORIC AND INTESTINAL OBSTRUCTION.

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(Received for publication, March 12, 1923.)

In a study of the blood of dogs after experimental intestinal obstruction,[1] it has been shown that there is characteristically a fall in chlorides and usually a rise in the CO_2-combining power of the plasma. It has been found that the increase in non-protein nitrogen noted by Tileston and Comfort,[2] Whipple,[3] and others does not begin until there is marked depletion of the blood chlorides. It has been shown also that the chemical changes in the blood after pyloric obstruction are identical with those following high intestinal obstruction.[4] Here the rise in non-protein nitrogen is more marked and the toxemia more severe, due seemingly to a greater loss of chlorides through vomiting.

The loss of chlorides after obstruction is not accounted for by the loss through vomiting. A fall in chlorides and rise in CO_2 occurred in one patient in the entire absence of vomiting, in rabbits, which cannot vomit, and repeatedly in dogs in the absence of any significant loss of chlorides in the vomitus.

The increase in CO_2-combining power of the plasma is due, we believe, to the utilization of the chlorine ion with the formation of sodium bicarbonate as a by-product. The level of sodium bicarbonate in the blood is variable and does not necessarily follow the course of the chlorides. The amount present in the plasma at any

[1] Haden, R. L., and Orr, T. G., *J. Exp. Med.*, 1923, xxxvii, 365.

[2] Tileston, W., and Comfort, C. W., Jr., *Arch. Int. Med.*, 1914, xiv, 620.

[3] Cooke, J. V., Rodenbaugh, F. H., and Whipple, G. H., *J. Exp. Med.*, 1916, xxiii, 717.

[4] Haden, R. L., and Orr, T. G., *J. Exp. Med.*, 1923, xxxvii, 377.

time will depend upon several factors. The most important are (1) the rate of formation of bicarbonate; (2) the rate of excretion; (3) the neutralizing effect on the bicarbonate of acid bodies formed in the course of the intoxication; and (4) the availability of sodium chloride from which bicarbonate may be formed.

The significance of the loss in chlorides has been discussed elsewhere.[1] It is known that the toxemia of intestinal obstruction is due to the action of some toxic agent, the source and nature of which are a matter of dispute. The poison causes a marked destruction of tissue protein. The clinical symptoms of toxemia parallel the tissue destruction. It is a question whether the toxemia may not be due to the absorption of the products of protein destruction rather than to the primary substance responsible for the protein destruction. The chlorides fall and are well depleted before there is evidence of much tissue destruction. This fact, together with the results of treatment, makes it seem probable that the behavior of the chlorides is in some way related to the action of primary toxic substances and that the chlorides have essentially a protective function. One conception of a protective action of the sodium chloride may be illustrated as follows:

$$\underset{\text{(toxic body)}}{X} + NaCl + H_2CO_3 = NaHCO_3 + \underset{\text{(non-toxic body)}}{X \cdot HCl}$$

The utilization of the chloride ion as a protective measure might be explained in other ways, such as the formation of chlorine substitution products of the toxic body or bodies. It seems possible also that the chlorine ion or hydrochloric acid might be utilized by the cells as a means of defense.

If such conception be correct the sodium chloride is concerned primarily with the body responsible for the tissue destruction. When sodium chloride is provided after pyloric or intestinal obstruction the blood should show only a utilization of the chlorides and a rise in CO_2-combining power of the plasma with no increase in non-protein nitrogen.

The following experiments have been made to determine the effect of furnishing an ample supply of sodium chloride on the toxemia of pyloric and intestinal obstruction.

Method.

The animals have been kept in metabolism cages and given no food during the course of the experiment. All operations were done under ether anesthesia with aseptic technique. Water was allowed *ad libitum*. Blood for chemical analysis was obtained from the jugular vein before operation and at 24 hour intervals thereafter, until the experiment ended with the recovery or death of the animal.

The duodenum was obstructed by section about 8 inches from the pylorus and inversion of the cut ends. The obstruction was thus complete in every instance. No difficulty was experienced with peritonitis. The pyloric obstruction was accomplished by ligation with heavy tape. This could be easily released without danger of infection. When the pylorus was thus ligated, the obstruction was never found to be complete at autopsy. An opening large enough to allow insertion of a lead pencil was uniformly present.

The non-protein nitrogen, sugar, and creatinine have been determined by the method of Folin and Wu;[5] the CO_2-combining power by the method of Van Slyke and Cullen;[6] urea nitrogen by the Van Slyke and Cullen modification of the Marshall method;[7] amino-acid nitrogen by the method of Folin;[8] uric acid by Benedict's method;[9] the blood chlorides on the tungstic acid filtrate in the manner suggested by Gettler;[10] and the chlorides in the urine by a modified Volhard-Arnold method.

EXPERIMENTAL.

We have found that untreated pyloric obstruction is uniformly fatal in the dog. The animals die in 4 days on an average. All show a rapid fall in blood chlorides, an increase in the CO_2-combining power of the plasma, and a rise in non-protein nitrogen in the blood.

Two dogs with the pylorus obstructed were given 50 cc. of 10 per cent sodium chloride solution subcutaneously at operation and daily thereafter. One dog lived 3 days, the other 4. The blood findings are shown in Table I. One dog showed no rise in non-protein nitrogen. Even with the large amount of salt given there was no rise

[5] Folin, O., and Wu, H., *J. Biol. Chem.*, 1919, xxxviii, 81.
[6] Van Slyke, D. D., and Cullen, G. E., *J. Biol. Chem.*, 1917, xxx, 289.
[7] Van Slyke, D. D., and Cullen, G. E., *J. Am. Med. Assn.*, 1914, lxii, 1558.
[8] Folin, O., *J. Biol. Chem.*, 1922, li, 377.
[9] Benedict, S. R., *J. Biol. Chem.*, 1922, li, 187.
[10] Gettler, A. O., *J. Am. Med. Assn.*, 1921, lxxvii, 1652.

in chlorides until shortly before death. The second dog showed some rise in non-protein nitrogen 48 hours after operation, but a later return to normal. Both dogs showed an elevation of CO_2-combining power of the plasma. The other constituents of the blood showed no signifi-

TABLE I.

Obstruction of the Pylorus. Blood Findings during Treatment with 10 Per Cent Sodium Chloride.

Dog No.	Day after operation.	Blood. Amount per 100 cc. Total non-protein nitrogen.	Urea nitrogen.	Uric acid.	Creatinine.	Amino-acid nitrogen.	Chlorides.	CO_2-combining power.	Urine. Chlorides.	Treatment.
		mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*vol. per cent*	*gm.*	
1	0	37.0	14.1	1.1	1.9	6.2	460	36.2		50 cc. of 10 per cent NaCl subcutaneously.
	1	34.5	12.6	1.6	1.1	5.7	460	43.8	7.2	" "
	2	38.0	23.4	1.3	1.2	5.4	410	40.0	2.8	100 cc. of 10 per cent NaCl subcutaneously.
	3	38.8	19.7		1.4	5.6	540	43.8	2.8	50 cc. of 10 per cent NaCl subcutaneously.
	4	35.3	13.3			5.6	630	47.5	0.40	" "
2	0	26.5	7.9	2.2	1.2	7.0	460	39.0		50 cc. of 10 per cent NaCl subcutaneously.
	1	35.3	12.1	1.4	1.3	5.7	480	48.5		" "
	2	36.5	14.9	1.1	1.4	5.3	480	52.2	1.71	" "
	3	35.3	14.0	1.4	1.3	7.4	630	64.5	0.27	(Died following operation for relief of obstruction.)
	4								0.45	

cant change. A very small amount of chloride was excreted in the urine and vomitus.

The obstruction at the pylorus was released in four dogs 48 to 72 hours after the initial operation. The blood findings are given in Table II. Two dogs died within 24 hours after the second opera-

TABLE II.

Blood Findings after Obstruction of the Pylorus and Later Release. Treatment of One Dog with Dry Sodium Chloride by Mouth.

Dog No.	Day after operation.	Blood. Amount per 100 cc. Total non-protein nitrogen.	Urea nitrogen.	Uric acid.	Creatinine.	Amino-acid nitrogen.	Chlorides.	CO_2-combining power.	Urine. Chlorides.	Treatment.
		mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*vol. per cent*	*gm.*	
3	0	22.4	8.9		1.2	5.0	490	30.5		
	3	114.0	62.6	1.4	1.2	6.5	440	48.5	0.09	Obstruction released.
	4	213.0	133.1	1.1	1.4	5.8	280	37.2	0.75	
										Died.
4	0	30.6	12.1		1.3		470	37.2		
	1	47.5	17.7		1.3		360	52.2	0.20	
	2	78.0	32.7		1.3		310		0.18	Obstruction released.
	3	208.0	117.7				280			
										Died.
5	0	23.0	7.9	2.3	1.3	5.0	420	34.3		
	1	51.8	10.7	3.5		6.5	350	57.0	0.30	
	2	71.3	38.3	3.8	1.8	7.1	240	67.3	0.36	Obstruction released.
	3	99.0	61.6	1.2	1.5	7.0	240	72.1	0.02	
	4	54.0	29.9	2.4	1.5	6.0	260	60.7		
	5	58.5	34.3	2.2	1.6	5.8	300	68.7	0.02	
	6	47.6	25.2		1.3		250	58.9	0.02	
	7		57.4					51.3	0.01	
	8	87.3	43.4	1.5	1.2	6.6	280	47.5	0.02	
	9	98.8	63.7	0.8	1.9	6.8	280	61.7	0.02	
	10		98.1						0.01	
										Died.
6	0	51.8	26.2	2.3	1.7	7.7	430	42.1		
	1	30.3	12.6	2.2	1.3	6.4	340	67.1	6.00	
	2	68.2	43.3		1.9	6.2	210	72.7	3.36	Obstruction released.
	3	132.2	72.9	2.8	2.0	8.7	260	74.5	2.00	
	4	83.3	57.1	2.7	1.5	8.8	230	80.1	0.55	
	5	77.3	38.2	2.8	1.7	10.4	270	74.5	0.38	
	6	107.0	63.9	2.9	2.0	9.3	260	74.5	0.18	
	7	158.0	98.8	2.0	2.3	8.4	350	56.9	0.15	
	8	208.0	123.3	2.1	2.7	7.9	290	58.8	0.21	
	9	173.0	100.8	1.7	2.5	7.1	250	67.5	0.12	
	10	230.0	141.5	1.8	2.5	7.7	270	65.3	0.20	
	11	250.0	159.7	1.9	2.3	8.0	270	71.8		10 gm. of NaCl by mouth.
	12	136.0	78.5	1.4	1.8	5.9	410	61.6	2.8	
										Died following intravenous injection of 25 per cent NaCl.

tion. The non-protein nitrogen continued to rise after the operation for the release of the obstruction. Two animals survived, but with a high level of non-protein nitrogen in the blood, a high nitrogen excretion in the urine, low blood chlorides, and a marked alkalosis. This seems to indicate that even with a relief of the obstruction, the toxic process continues and the animal is unable to recover its chemical equilibrium. The dogs surviving showed little healing of the wounds. One (No. 5) died of peritonitis arising in a wound infection on the 13th day. The other (No. 6) on the 11th day showed a non-protein nitrogen of 250 mg. per 100 cc. of blood, chloride of 270 mg., and CO_2 of 72 volumes per cent. At this time 10 gm. of dry sodium chloride were given by mouth. The following morning the non-protein nitrogen had dropped to 136 mg., the CO_2-combining power was down to 61.6 mg., and the chlorides had risen to 410 mg. Unfortunately, at this point the animal died following an intravenous injection of 25 per cent sodium chloride solution.

Two dogs with pyloric obstruction had the obstruction released 72 hours after the primary operation. One was given 50 cc. of distilled water, the other 50 cc. of 25 per cent glucose solution subcutaneously daily. Both dogs showed a rapid rise in non-protein nitrogen in marked contrast to those given a like amount of 10 per cent sodium chloride solution (Table I). The blood findings are given in Table III. At the time of release of the obstruction one dog had a non-protein nitrogen of 105 mg. per 100 cc. of blood, the other of 76 mg. Both were given 50 cc. of 10 per cent sodium chloride solution subcutaneously at the time of the second operation. There was a rapid return of the blood to normal and complete recovery without further treatment. Both dogs showed a slight secondary rise in non-protein nitrogen. This rise has been observed in other treated animals and also in two patients. There is no obvious explanation for it.

Untreated duodenal obstruction in the dog is uniformly fatal.[1] The duration of life is 4 days on an average, although some animals live as long as 9 days. They show in the blood the same changes as those described as characteristic of pyloric obstruction.

Two dogs with the duodenum obstructed by section and inversion of the cut ends were treated after the obstruction had existed for 48 hours. The results are shown in Table IV. In one animal (No. 9)

TABLE III.

Obstruction of the Pylorus and Later Release. Blood Findings during Treatment with Water, Glucose Solution, and 10 Per Cent Sodium Chloride.

Dog No.	Day after operation.	Blood. Amount per 100 cc. Total non-protein nitrogen.	Urea nitrogen.	Uric acid.	Creatinine.	Amino-acid nitrogen.	Chlorides.	CO_2-combining power.	Urine. Chlorides.	Treatment.
		mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*vol. per cent*	*gm.*	
7	0	36.1		1.1	1.2	7.4	450	42.8		50 cc. of distilled water subcutaneously.
	1	33.3	12.6	1.1	1.2	7.2	470	51.3		" "
	2	36.1	17.7		1.5	7.7	300	53.2	0.92	" "
	3	105.0	54.6	2.1	2.0	8.2	260	66.4	0.16	Obstruction released. Given 50 cc. of 10 per cent NaCl subcutaneously.
	4	37.5	14.0	2.1	2.0	7.7	570	64.5	0.60	
	5	21.8	7.5	3.3	1.2	6.0	430	50.4		
	7	55.5	28.0	1.5	1.4	7.0	470	42.8	0.87	
	8	20.8	7.0		1.3	5.0	380	48.5		
	9								0.06	
	10	22.2	7.0	1.3	1.3	4.3	450	53.2		
	11								0.98	
	12								0.81	
	13	28.2	6.5	1.4	1.1	4.4	510	47.5		
8	0	25.4	9.3		1.8	6.2	590			50 cc. of 25 per cent glucose subcutaneously.
	1	29.1	11.7		1.8		590	40.0	0.01	" "
	2	31.9	17.5	1.6	1.5	6.6	340	59.8		" "
	3	76.5	52.5	1.6	1.5	6.7	260	53.8	1.2	Obstruction released. 50 cc. of 10 per cent NaCl subcutaneously.
	4	30.0	13.1	1.6	1.3	5.8	390	63.6	1.0	
	5	22.4	7.5	0.9	1.3	5.0	410	47.5	1.9	
	6	53.3	22.9	1.3	1.4	4.8	430	51.3	0.07	
	7	41.6	16.8	1.6	1.3	4.1	410	51.3		
	8	24.0	12.1	1.9	1.3	4.3	430	55.1	0.08	
	10	21.4	7.9		1.4	4.0	460	55.1		

the non-protein nitrogen had risen from 23 to 54 mg. per 100 cc. of blood. 50 cc. of 10 per cent sodium chloride solution were given subcutaneously daily for 2 days. The non-protein nitrogen returned

TABLE IV.

Obstruction of the Duodenum. Blood Findings during Treatment with Sodium Chloride.

Dog No.	Day after operation.	Blood. Amount per 100 cc. Total non-protein nitrogen.	Urea nitrogen.	Uric acid.	Creatinine.	Amino-acid nitrogen.	Chlorides.	CO_2-combining power.	Urine. Chlorides.	Treatment.
		mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*vol. per cent*	*gm.*	
9	0	22.7	5.6	1.4	1.2	6.6	430	35.3		
	1	30.0	8.4	1.8	1.2	7.6	340	58.9	5.4	
	2	53.5	24.3	1.7	1.3	7.7	300	47.5	0.03	50 cc. of 10 per cent NaCl subcutaneously.
	3	32.3	17.8	2.0	1.5	6.8	370	63.6		" "
	4	36.1	10.5	1.7	1.0	9.3	440	41.9	0.7	Died following lateral anastomosis for relief of obstruction.
	5								1.37	
10	0	26.1	6.0	1.3	1.5	6.1	470	43.8		
	1	28.5	9.3	1.9	1.5	6.6	410	45.7	1.91	
	2	47.6	21.0	1.7	1.7	6.2	360	52.2	2.20	
	3	115.0	65.2	1.9	1.7	7.1	290	55.1	0.71	500 cc. of 3 per cent NaCl subcutaneously.
	4	72.8	34.4	2.5	1.5	6.1	500	67.3	1.48	500 cc. of 2 per cent NaCl subcutaneously.
	5	50.0	17.8		1.5	7.0	550	42.8	2.98	

to normal within 48 hours after the treatment was begun. The dog died following a lateral anastomosis for relief of the obstruction. The second dog (No. 10), when the non-protein nitrogen had risen to 115 mg. per 100 cc. of blood, was given 500 cc. of 3 per cent sodium chloride subcutaneously and 500 cc. of 2 per cent sodium chloride

TABLE V.

Obstruction of the Duodenum. Blood Findings during Treatment with Physiological Salt Solution.

Dog No.	Day after operation	Blood. Amount per 100 cc. Total non-protein nitrogen.	Urea nitrogen.	Uric acid.	Creatinine.	Amino-acid nitrogen.	Chlorides.	CO_2-combining power.	Urine. Chlorides.	Treatment.
		mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*vol. per cent*	*gm.*	
11	0	22.5	7.9		1.4	6.5	470	34.3		500 cc. of 0.85 per cent NaCl subcutaneously.
	1	27.3	11.2	2.3	1.5	6.6	430	40.0	0.34	" "
	2	34.1	13.5	2.5	1.4	7.1	360	54.1	3.78	" "
	3	50.0	25.2	3.0	1.4	6.9	360	64.5	6.41	" "
	4	37.5	14.0	1.4	1.4	7.0	370	66.4	6.42	" "
	5	33.1	12.6	1.5	1.2	6.5	380	75.8	1.20	" "
	6	35.7	15.9	3.3	1.3	6.4	370	83.4	1.24	" "
	7	35.7	14.0	2.2	1.7	6.1	380	81.5	0.56	" "
	8	33.1	14.0	2.3	1.7	6.3	400	70.2	1.29	" "
	9	24.4	9.3	2.4	1.5	6.3	410	74.0	2.24	" "
	10	41.1	18.6	2.0	1.6	6.3	360	80.6	4.83	" "
	11	32.3	13.5	3.1	1.5	6.3	350	89.2	3.00	" "
	12	30.0	13.1	2.7	2.2	6.3	360	86.2	4.53	" "
	13	35.7	10.5		1.9	5.6	380	84.3	1.45	" "
	14	37.5	17.8	2.0	1.7	5.4	430	91.3	2.39	" "
	15	34.1	15.9	2.1	1.8	5.4	400	84.3	6.75	" "
	16	36.4	14.0	2.3	1.6	5.6	400	77.7	1.75	" "
	17	31.6	12.6	2.5	1.7	5.6	470	70.2	2.44	" "
	18								0.98	" "
	19	25.2	10.7	2.6	1.3	4.9	540	49.4	1.64	" "
	20								0.45	" "
	21								0.97	" "
12	0	31.6	11.7	1.5	1.4	6.2	450	37.2		" "
	1	30.6	11.2	1.7	1.4	6.1	420		3.34	" "
	2	31.9	11.7	1.7	1.5	6.7	400	59.8	3.43	" "
	3	31.6		1.5	1.9	5.5	340	66.4	3.23	" "
	4	33.3	17.8	2.3	1.8	6.0	350	84.3	2.22	" "
	5	27.3	12.1		1.5	5.3	350	73.0	4.45	" "
	6	31.6	9.3		1.4	5.8	360	73.0	1.62	" "
	7	30.0	10.7	1.5	1.8	5.8	380	69.2	1.61	" "
	8								3.22	" "

TABLE V—*Concluded.*

Dog No.	Day after operation.	Blood. Amount per 100 cc. Total non-protein nitrogen.	Urea nitrogen.	Uric acid.	Creatinine.	Amino-acid nitrogen.	Chlorides.	CO_2-combining power.	Urine. Chlorides.	Treatment.
		mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*vol. per cent*	*gm.*	
12	9	30.0	14.5	3.1	1.3	6.3	370	74.9	1.83	500 cc. of 0.85 per cent NaCl subcutaneously.
	10								0.08	" "
	11	25.0	7.0	3.3	1.3	5.1	380	73.0	2.80	" "
	12	26.5	12.6	3.1	1.4	5.6	350	88.2	2.40	" "
	13	25.0	10.7	3.3	1.3	4.8	360	90.2	0.07	" "
	14	26.5	11.2	3.4	1.4	5.6	330	80.6	1.92	" "
	15	26.1	10.3	2.1	1.3	4.8	310	83.4	9.94	" "
	16	26.1	14.0	3.1	1.4	5.3	360	82.4	2.56	" "
	17	28.5	11.7		1.5	5.9	370	86.5	0.01	" "
	18	35.3	14.0		1.6	5.0	400	50.0	1.98	" "
	19	31.6	7.0			6.1	390	70.2	8.52	" "
	20	30.0	14.5		1.7	5.7	400	67.6	3.21	" "
	21	37.5	14.5		1.8	4.7	440	68.3	0.22	" "
	22	20.0	9.8		2.0			67.3	1.54	" "
	23	24.4	9.8		1.8		440	67.3	1.78	" "
	24	27.8	7.0		1.5	4.3	390	74.9	0.30	" "
	25	29.1	14.0		1.4	4.4	420	84.3	6.53	" "
	26	27.3	12.2		1.1	4.2	440	80.6	0.67	" "
	27	44.1	20.6		1.1	4.5	440	86.2	0.20	" "

the following day. The non-protein nitrogen fell to 50 mg. The animal died before an operation for relief of the obstruction was done.

Two dogs with the duodenum obstructed by section and inversion of the cut ends were given 500 cc. of 0.85 per cent sodium chloride subcutaneously on the day of operation and each day thereafter until death. One lived 21 days, the other 28. The blood findings are given in Table V. One animal (No. 11) showed an increase in the non-protein nitrogen on the 3rd day after operation. Otherwise, this was practically within normal limits. The chloride of the blood

remained below normal and there was a well marked alkalosis. The other animal (No. 12) showed a normal non-protein nitrogen throughout. Determination of the chlorides and CO_2-combining power gave results similar to those obtained in Dog 11. Dog 11 was given

TABLE VI.

Obstruction of the Duodenum. Blood Findings during Treatment with Distilled Water.

Dog No.	Day after operation.	Blood. Amount per 100 cc. Total non-protein nitrogen.	Urea nitrogen.	Uric acid.	Creatinine.	Amino-acid nitrogen.	Chlorides.	CO_2-combining power.	Urine. Chlorides.	Treatment.
		mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*vol. per cent*	*gm.*	
13	0	31.2	10.3	1.5	1.3	7.7	490	29.6		500 cc. of distilled water subcutaneously.
	1	33.7	10.7	1.6	1.3	6.4	390	40.0	1.15	" "
	2	37.2	14.5	2.5	1.3	6.6	340	50.4	0.08	" "
	3								2.31	Died during night.
14	0	28.5	10.3	1.7	1.6	6.6	440	36.2		500 cc. of distilled water subcutaneously.
	1	37.5	17.3		2.0		340	38.1	0.80	" "
	2								1.12	
15	0	27.0	9.3	3.0	1.3	6.3	430	40.0		500 cc. of distilled water subcutaneously.
	1	35.3	12.6	3.0	1.4	6.6	370	47.5	0.40	" "
	2	37.5	13.0	2.8	1.4	6.2	360	39.0	0.23	" "
	3	55.5	23.4	3.0	1.3	7.1	330	45.7	0.34	" "
	4								0.19	

89.25 gm. of NaCl and excreted 53.67 gm. Dog 12 was given 119.00 gm. and excreted 89.25 gm.

One animal died of a terminal peritonitis from the rupture of an intussuscepted ileum. The other showed at autopsy an extensive intussusception of the ileum with extreme ulceration.

TABLE VII.

Obstruction of the Duodenum. Blood Findings during Treatment with Solutions of Glucose and of Sodium Bicarbonate.

Dog No.	Day after operation.	Blood. Amount per 100 cc. Total non-protein nitrogen.	Urea nitrogen.	Uric acid.	Creatinine.	Amino-acid nitrogen.	Chlorides.	CO_2 combining power.	Urine. Chlorides.	Treatment.
		mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*vol. per cent*	*gm.*	
16	0	36.1	13.5		1.6	6.1	410	30.5		500 cc. of 2 per cent glucose subcutaneously.
	1	44.1	15.9	2.0	1.5	5.4	350	51.3	4.9	" "
	2	75.5	32.7	2.0	1.4	7.0	300	58.9	3.95	" "
	3	94.0	44.7	2.0	1.4	7.2	260	55.1	0.98	" "
	4								0.95	Died during night.
17	0	33.3	7.9		1.8	6.3	450	34.3		500 cc. of 1 per cent $NaHCO_3$ subcutaneously.
	1	32.3	11.2	1.8	1.4	5.8	350	51.3	0.79	" "
	2	48.1	19.2	2.3	1.5	5.2	320	60.7	2.04	" "
	3	57.5	28.7	3.3	1.4	5.6	270	65.5	0.26	" "
	4	44.1	13.3		1.4	5.4	280	79.2	1.70	" "
	5	37.5	11.2	2.7	1.4	5.0	220	96.2	0.22	" "
	6	50.0	24.3	3.0	1.4	6.3	210	84.3	1.00	" "
	7	50.5	22.0	3.3	1.4	5.6	200	93.4	0.02	" "
	8	62.5	36.4		1.3	5.8	190	103.4		" "
	9	62.5	36.4		1.6	5.9	200	110.0	0.12	" "
	10	88.3	41.3		1.6	5.9	240	110.0	0.12	" "
	11								0.33	Died during night.
18	0	34.1		4.1	1.4	7.9	470	21.1		500 cc. of 1 per cent $NaHCO_3$ subcutaneously.
	1	45.3	20.1	3.0	1.5	6.6	360	49.4	1.41	" "
	2	57.0	29.4	3.2	1.9	7.0	330	55.1	0.38	" "
	3	39.5	16.4		1.3		330	63.6	0.81	" "
	4	72.8	39.2	4.8	1.7	6.8	320	78.7	0.29	" "
	5	76.5	35.0	5.8	1.0	6.8	260	80.6	0.45	" "
	6	120.0	30.4	4.8	1.1	6.8	230	82.5	1.23	" "
	7	162.0	65.9	9.3	1.5	9.3	200	47.5	2.56	" "
	8								0.22	
19	0	35.3	14.7		1.9		470	36.2		500 cc. of 1 per cent $NaHCO_3$ subcutaneously.
	1	46.0	10.3		1.9	8.2	410	36.2	6.39	" "
	2	120.0	54.3		1.9	10.3	320	47.5	7.68	" "

The question at once arose as to whether the effects obtained were due to the fluid alone, diuresis, or simply to some salt action. Three dogs after duodenal obstruction were given 500 cc. of distilled water subcutaneously each day. The blood findings are shown in Table VI. One animal lived 24 hours, one 48 hours, and the third 72 hours. It is obvious that fluid alone cannot account for the results obtained with physiological salt solution. The animals died more quickly than control animals given no treatment.

One dog was given 500 cc. of 2 per cent glucose solution subcutaneously daily. There was a rapid rise in non-protein nitrogen and death in 72 hours. Three dogs were given 500 cc. of 1 per cent sodium bicarbonate solution subcutaneously daily. One lived 10 days, the other 7 days, and the third 48 hours. The blood findings are shown in Table VII. The changes in the blood are similar to those found in untreated animals, except that the alkalosis was more marked. The two animals living longest showed extreme muscle stiffness, coarse muscle tremors, slow respiration, and purulent conjunctivitis with ulceration of the cornea.

DISCUSSION.

These experiments show that the administration of sodium chloride has a marked effect on the changes in the blood taking place after pyloric and intestinal obstruction in the dog. The toxemia can be prevented if the chloride be given at the onset of obstruction. Chloride is likewise valuable in combating the toxemia when given after the obstruction has existed long enough to produce marked chemical changes in the blood.

Since water alone and solutions of glucose or sodium bicarbonate have little or no effect in preventing the characteristic changes in the blood following pyloric or intestinal obstruction, the findings indicate that the sodium chloride is the active agent in the experiments reported. Excellent therapeutic results have been obtained when the chloride is given dry by mouth or in a concentrated solution.

It is, of course, necessary that an abundant supply of fluid be furnished. The fact is striking that dogs provided merely with fluid die more quickly than untreated animals. We have used the

concentrated solution of sodium chloride simply to prove that the good results obtained are referable to this salt.

The chemical changes in the blood of dogs given physiological salt solution from the onset of obstruction are especially important. The administration of the solution appeared to prevent the protein destruction. However, a large portion of the chlorides was used up in some manner, and, simultaneously, alkalosis developed. These findings suggest that the presence of the chloride in some way affects the action of a primary toxic substance responsible for the nitrogen destruction. There is much to indicate that the toxemia is due to secondary products of protein destruction and not directly to the primary toxic body.

After the toxemia of obstruction is well developed, treatment is more difficult. At this time the chlorides are depleted. The object of the treatment should be to restore chlorides quickly and to maintain them at a normal level, while furnishing the maximum amount of fluid which the organism can handle. It is difficult to accomplish these ends with physiological saline, since about 1 gm. of sodium chloride per kilo of body weight must be given as an initial treatment.

The glucose solutions were of no specific value in combating the toxemia. Mayo[11] in a recent summary of the treatment of intestinal obstruction in man suggests only the use of glucose solution. The results of sodium bicarbonate administration in the present experiments are especially interesting. Dogs given sodium bicarbonate lived no longer and were in much worse physical condition at death than untreated animals.

Wound healing was good in animals given the chloride and poor in the other animals.

Hartwell and Hoguet showed in 1912[12] that a dog with complete duodenal obstruction could be kept alive for 3 weeks if the fluid lost by vomiting and by way of the kidneys was replaced with physiological salt solution. The control dogs died in 3 to 10 days. They considered these results as proof of their theory that the toxic symptoms of intestinal obstruction are due to tissue dehydration. They

[11] Mayo, W. J., *Surg., Gynec. and Obst.*, 1923, xxxvi, 112.

[12] Hartwell, J. A., and Hoguet, J. P., *J. Am. Med. Assn.*, 1912, lix, 82.

gave the saline solution simply as a means of furnishing an isotonic fluid. They made no chemical studies on the blood or estimations of the chloride excretion in the urine. The excellent results obtained by them were no doubt due to the specific action of the sodium chloride.

Similarly MacCallum and his associates[13] have shown that the symptoms following obstruction of the pylorus can be prevented by furnishing a large supply of chlorides. However, they attribute the symptoms to a loss of chlorides through vomiting, with alkalosis as a result. The chlorides were given merely to replace those so lost.

It should be emphasized that our purpose in giving the chloride is different from that of the investigators quoted. We believe that in the sodium chloride we are furnishing a direct protective factor.

It seems possible that other chlorides, other salts or combination of salts, may have a similar, perhaps better action. Further studies are under way to settle this point.

SUMMARY AND CONCLUSIONS.

Experiments to determine the effect of furnishing an ample supply of sodium chloride on the toxemia of pyloric and intestinal obstruction are reported.

A fall in chlorides is the first and seemingly most significant change to take place in the blood after pyloric and intestinal obstruction.

The chloride is apparently utilized by the body as a protective measure against the primary toxic substance.

Two dogs with pyloric obstruction were given 50 cc. of 10 per cent NaCl subcutaneously daily. One lived 3 days, the other 4. The blood showed little change, except a marked terminal rise in chlorides. Animals given a like amount of distilled water or 25 per cent glucose showed the changes typical of untreated animals.

The obstruction of the pylorus was released in six dogs 48 to 72 hours after the initial operation. Two died within 24 hours after the second operation with a high non-protein nitrogen in the blood. Two survived but showed a high level of non-protein nitrogen in the blood and a high nitrogen excretion in the urine, low blood chlorides, and a marked alkalosis. One dog in such a state died on the 13th day

[13] MacCallum, W. G., Lintz, J., Vermilye, H. N., Legett, T. H., and Boas, E., *Bull. Johns Hopkins Hosp.*, 1920, xxxi, 1.

from peritonitis, arising in a wound infection. The other showed a marked fall in non-protein nitrogen in the blood following the administration of 10 gm. of sodium chloride by mouth, but died following the intravenous injection of 25 per cent sodium chloride. Two animals were given 50 cc. of 10 per cent NaCl subcutaneously, at the time of the second operation. The blood rapidly returned to normal and complete recovery followed.

Two dogs with the duodenum obstructed by section and inversion of the cut ends were treated with 10 per cent sodium chloride after the obstruction had existed for 48 hours and the characteristic blood changes had developed. The non-protein nitrogen returned to normal within 48 hours after treatment was begun. One dog died following a lateral anastomosis for relief of the obstruction. A second operation was not attempted in the other animal.

Two dogs in which the duodenum was obstructed by section and inversion of the cut ends were given 500 cc. of 0.85 per cent NaCl subcutaneously on the day of operation and each day thereafter until death. One dog lived 21 days, the other 28. Both dogs showed a marked alkalosis, but never any rise in the non-protein nitrogen of the blood. The animals at autopsy showed intussusception of the ileum with extensive ulceration. In one there was a perforation and terminal peritonitis. The operation wounds healed normally.

Three dogs with section of the duodenum were given 500 cc. of distilled water every day. One died in 24 hours, one in 48 hours, and the third in 72 hours. Autopsy showed no cause for death other than toxemia.

One dog with section of the duodenum was given 500 cc. of 2 per cent glucose every day. The blood showed a rapid rise in non-protein nitrogen and carbon dioxide-combining power, and a fall in chlorides. The animal died 72 hours after operation.

Three dogs with section of the duodenum were given 500 cc. of 1 per cent sodium bicarbonate every day. One dog died in 72 hours, one lived 7 days, and the third lived 9 days. All developed a high non-protein nitrogen in the blood and two showed marked clinical symptoms of an alkalosis.

These results demonstrate that solutions of sodium chloride have a marked effect in preventing and controlling the toxemia of pyloric

and intestinal obstruction as shown in clinical symptoms and in chemical changes in the blood.

Dogs given an abundant supply of distilled water died more quickly than untreated control animals. Solutions of glucose have no specific value, and sodium bicarbonate solutions prolong life only a short while. Good therapeutic results have been obtained with very concentrated sodium chloride solutions, and with dry sodium chloride given by mouth.

It seems evident that sodium chloride has a specific action in preventing and possibly in controlling the changes produced by the toxic body.

Sodium chloride is a valuable therapeutic agent in pyloric and high intestinal obstruction.

THE SOLUBLE SPECIFIC SUBSTANCE OF PNEUMOCOCCUS.

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In 1917 Dochez and Avery (1) showed that whenever pneumococci are grown in fluid media, there is present in the cultural fluid a substance which precipitates specifically in antipneumococcus serum of the homologous type. This soluble substance is demonstrable in culture filtrates during the initial growth phase of the organisms; that is, during the period of their maximum rate of multiplication when little or no cell death or disintegration is occurring. The formation of this soluble specific material by pneumococci on growth *in vitro* suggested the probability of an analogous substance being formed on growth of the organism in the animal body. Examination of the blood and urine of experimentally infected animals gave proof of the presence of this substance in considerable quantities in the body fluids following intraperitoneal infection with pneumococcus. In other words, this soluble material elaborated at the focus of the disease readily diffuses throughout the body, is taken up in the blood, passes the kidney, and appears in the urine unchanged in specificity. Similarly, a study of the serum of patients suffering from lobar pneumonia has revealed a substance of like nature in the circulating blood during the course of the disease in man. Furthermore, examination of the urine of patients having pneumonia due to pneumococci of Types I, II, and III has shown the presence of this substance in some stage of the disease in approximately two-thirds of the cases. Recently from filtered alkaline extracts of pulverized bacteria of several varieties, including pneumococci, Zinsser and Parker (2) have prepared substances which appear free from coagulable protein. These substances, called "residue antigens,"

are specifically precipitable by homologous antisera. These observers consider these acid- and heat-resistant antigenic materials analogous to the soluble specific substance of pneumococcus described by Dochez and Avery (1). In spite of the fact that these "residue antigens" are precipitable by homologous sera produced by immunization with the whole bacteria, Zinsser and Parker have so far failed to produce antibodies in animals by injecting the residues.

In the earlier studies by Dochez and Avery certain facts were ascertained concerning the chemical characteristics of this substance. It was found that the specific substance is not destroyed by boiling; that it is readily soluble in water, and precipitable by acetone, alcohol, and ether; that it is precipitated by colloidal iron, and does not dialyze through parchment; and that the serological reactions of the substance are not affected by proteolytic digestion by trypsin. Since the substance is easily soluble, thermostable, and type-specific in the highest degree, it seemed an ideal basis for the beginning of a study of the relation between bacterial specificity and chemical constitution. The present report deals with the work done in this direction.

EXPERIMENTAL.

The organism used in the present work was Pneumococcus Type II. The most abundant source of the soluble specific substance appeared to be an 8 day autolyzed broth culture; hence this material was used as the principal source of supply. For comparison dissolved pneumococci and lots of urine containing the specific substance were also worked up, with essentially the same results, as will be seen from Table I.

The process for the isolation of the soluble specific substance consisted in concentration of the broth, precipitation with alcohol, repeated re-solution and reprecipitation, followed by a careful series of fractional precipitations with alcohol or acetone after acidification of the solution with acetic acid, and, finally, repeated fractional precipitation with ammonium sulfate and dialysis of the aqueous solution of the active fractions.

Five lots of 15 liters each of 8 day cultures of Pneumococcus Type II in meat infusion phosphate broth are each concentrated on the water bath in large evapo-

rating dishes to 1,000 to 1,200 cc. and precipitated in a separatory funnel by the gradual addition, with vigorous rotation, of 1.2 volumes of 95 per cent alcohol. The mixture separates into two layers, and is allowed to stand over night, or for several hours. The upper layer, which is almost black and comprises the largest part of the mixture, contains only traces of the soluble specific substance, and is siphoned off and discarded. The lower, more viscous layer is run into a 250 cc. centrifuge bottle (occasionally a second will be required), capped, and rotated at high speed for ½ hour. Three layers are formed, of which the uppermost is merely a further amount of the liquid previously discarded. The middle layer consists of a compact, greenish cake of insoluble matter and gummy material, and contains most of the soluble specific substance. The bottom layer, from which salts often separate, is a brownish syrup rich in salts and nitrogenous matter and relatively poor in specific substance, and can, by careful manipulation, be poured off to a large extent. Although a small proportion of the specific substance is lost if this syrup is discarded, its elimination represents so considerable a purification as to warrant the sacrifice of the active material contained. The gummy cake remaining in the centrifuge bottle, together with adhering salts and syrup, is now rinsed out and ultimately combined with similar material from the other lots. All of this is then dissolved as completely as possible in water, care being taken to break up the many lumps of gummy material, diluted to 1 liter, and again precipitated with alcohol. In this case about 1.3 liters are required to precipitate all but the last traces of active material from the upper layer. This is again discarded and the lower layer treated as before. At this stage there is relatively less of the bottom layer, and it is more difficult to separate it from the cake containing the specific substance, but as much as possible is removed. The remaining material is smoothed out with water, diluted to about 500 cc., and centrifuged. The precipitate is washed twice with water, and the washings are combined with the main solution. The still turbid liquid, the volume of which should be about 750 cc., is put through the alcohol purification process a third time, about 1.1 liters of alcohol being required. After having been centrifuged, the active material is again dissolved in water, made definitely acid to litmus with acetic acid, and again centrifuged. The precipitate is washed three times with water acidulated with acetic acid, and the filtrate and washings are combined in a separatory funnel and diluted again if necessary to 750 cc. Acetone (redistilled) is now added until a permanent precipitate forms, about 250 cc. being necessary. The precipitate is allowed to settle, whereupon the lower part of the mixture containing the precipitate is drawn off and centrifuged. The clear supernatant fluid is restored to the main solution, while the precipitate, which consists largely of insoluble material and gives an aqueous solution almost devoid of activity, is discarded. Fractional precipitation is continued, and even when the specific substance appears in quantity in the precipitate, it is occasionally possible to separate a lower, inactive, syrupy layer, as in the previous purifications by alcohol. Addition of acetone is continued until a test portion, heated on the water bath to remove acetone, diluted with saline, and neutralized, no longer gives a pre-

cipitate with immune serum, after which the upper layer may be discarded. The active precipitates are then redissolved in water, centrifuged again, and the supernatant liquid is diluted to 375 cc., reacidified with acetic acid, and again fractionated with acetone. If inactive fractions are obtained, the process is again repeated until no further purification results. Alcohol may be used for these fractionations instead of acetone, the only difference being that a somewhat larger proportion is required. The active material is then dissolved in about 150 cc. of water and again made definitely acid with acetic acid. The solution is treated with solid ammonium sulfate until the first slight precipitate forms. This is generally inactive, and if so, may be discarded. Finally, ammonium sulfate is added to saturation, completely precipitating the specific substance if the volume of the solution is not too great. The mixture is allowed to stand for several hours and is then centrifuged and the precipitate washed with a little saturated ammonium sulfate solution. It is redissolved in about 75 cc. of water acidified with acetic acid, centrifuged if necessary, and again precipitated by saturation with ammonium sulfate. Finally, the specific substance so obtained is dissolved in water and dialyzed first against running tap water in the presence of chloroform and toluene, and finally against distilled water until tests for sulfate and phosphate ion are negative. Addition of acetic acid during the early stages of the dialysis assists in the removal of calcium, which otherwise forms a large part of the ash.

The dialyzed solution is concentrated to dryness on the water bath and the residue redissolved in hot water. If the solution is not perfectly clear, it is centrifuged again before being evaporated to dryness, and the whole process is repeated as long as insoluble material separates. Toward the end of the final concentration absolute alcohol may be added to assist in the precipitation of the substance.

Variations in the exact volumes given are often necessary with different lots of broth, but this will occasion little difficulty if all fractionations are controlled by the specific precipitin test.

As so obtained the soluble specific substance forms an almost colorless varnish-like mass which may be broken up and dried to constant weight at 100°C. *in vacuo*. The yield from 75 liters of broth averages about 1 gm., although it varies within rather wide limits in individual lots.

By the method outlined above all substances precipitable with phosphotungstic acid or capable of giving the biuret reaction were eliminated. The residual material (Preparation 17, in Table I), for which no claim of purity is made, as efforts at its further purification are still under way, contained, on the ash-free basis, 1.2 per cent of nitrogen. It was essentially a polysaccharide, as shown by the formation of 79 per cent of reducing sugars on hydrolysis, and by the isolation and identification of glucosazone from the products of hydrolysis.

0.4 gm. of Preparation 17 was heated under a reflux condenser with 40 cc. of 0.5 N HCl for 7 hours. The filtered solution was treated with 0.4 cc. of phenylhydrazine, followed by saturated sodium acetate solution until Congo red paper was no longer turned blue. After 1 hour in the water bath the crystalline osazone was filtered off, washed with water, purified by stirring well with dry methyl alcohol, and again filtered. 0.037 gm. was obtained, melting and decomposing at 196–197°C. with preliminary darkening and softening. A second fraction of 0.026 gm. of even purer osazone was obtained by heating the mother liquors of the first fraction 2 hours longer after addition of 0.2 cc. more of phenylhydrazine and purifying the crude product with methyl alcohol. This portion melted quite sharply at 205–206°. The true melting point of pure glucosazone is 208°.

50 mg. of the combined fractions, dissolved in 3 cc. of alcohol and 2 cc. of pyridine, gave, in a tube 0.5 dm. long, an initial rotation of −0.20° and a final rotation of −0.08°, while Levene and La Forge (3) give −0.62° and −0.35° for a solution of twice the concentration of recrystallized glucosazone. The only other hexose whose osazone rotates to the left is altrose, and this is known only as a laboratory product. Moreover, its osazone melts at 178° and decomposes at 189°.[1]

The aqueous solution of the substance gave the Molisch reaction out to the limit of delicacy of the test. Reduction of Fehling's solution occurred only after hydrolysis. Phosphorus was present only in traces; sulfur and pentoses were absent. A 1 per cent solution gave no biuret reaction, no precipitate with phosphotungstic acid, mercuric chloride, or neutral lead acetate, precipitated heavily with basic lead acetate, and gave a faint turbidity with tannic acid. Calcium is very tenaciously retained, but does not appear to be an essential part of the molecule, as the specific reaction was also given by calcium-free preparations. No color is given by iodine solution.

The soluble specific substance is remarkably stable to acids. A solution in 0.5 N hydrochloric acid maintained its activity undiminished and failed to reduce Fehling's solution after 36 hours at room temperature, but showed reducing sugars and absence of precipitation with immune serum after transfer to the water bath.

The limits of delicacy of the specific precipitation of the soluble substance were titrated, as shown in Table I, and it is seen that Preparation 17 still gave a specific reaction in a dilution as high as 1:3,000,000.

Table I represents a summary of the reactions of some of the earlier preparations worked with, as well as the later ones. Prep-

[1] We are greatly indebted to Dr. P. A. Levene for valuable aid and advice on this phase of the work.

aration 4 was obtained from the urine of a patient with a Type II pneumococcus infection, while No. 8 was obtained from an antiformin solution of the pneumococci. In both of these cases, as well as in Nos. 9, 11, and 15, the method of purification given above had not been fully worked out.

Attempts to stimulate antibody production by the immunization of animals with the purified substance yielded negative results.

TABLE I.

Summary of the Properties of Various Preparations of the Soluble Specific Substance of Pneumococcus Type II.

Preparation No.	Total N.	Hydrolysis.			S	P	Specific rotation $[\alpha]_D$	Precipitation with immune serum.*	Molisch reaction.†
		NH_2 N	NH_3 N	Reducing sugars.					
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*			
4‡	6.1	3.5			1.5		Too dark.	1:80,000	
4A§	4.7			63.0		1.0‖	−20.6°	1:640,000	1:320,000
8¶	2.9			++			+19.8°	1:1,250,000	1:640,000
9	6.6				1.8		−8.6°	1:640,000	
11	2.1	0.9	1.3				+31.6°	1:2,500,000	1:1,250,000
15	2.0			49.0		0.9‖	+30.8°	1:2,500,000	1:1,250,000
17	1.2	C = 46.2 per cent.	H = 6.1 per cent.	79.0	None.	Tr.	+55.7°	1:3,000,000	1:1,500,000

* After 2 hours at 37°C. and over night at 4°.

† Unless the α-naphthol solution is fresh, other colors will mask the purple at high dilutions. The figures represent the dilution of the preparation itself; in other words, the highest dilution giving the reaction in the previous column.

‡ From urine.

§ Preparation 4 repurified.

‖ Due to incomplete dialysis.

¶ From dissolved pneumococci.

DISCUSSION.

While it has long been known that the capsular material of many microorganisms consists, at least in part, of carbohydrates (4), any connection between this carbohydrate material and the specificity relationships of bacteria appears to have remained unsuspected. While it cannot be said that the present work establishes

this relationship, it certainly points in this direction. Evidence in favor of the probable carbohydrate nature of the soluble specific substance is the increase in specific activity with reduction of the nitrogen content, the increase in optical rotation with increase in specific activity, the parallelism between the Molisch reaction and specific activity, the high yield of reducing sugars on hydrolysis, and the actual isolation of glucosazone from a small quantity of the material. The small amounts of substance available up to the present have hindered the solution of the problem, and it is hoped that efforts at further purification of the soluble specific substance, now in progress with larger amounts of material, will definitely settle the question.

SUMMARY.

1. A method is given for the concentration and purification of the soluble specific substance of the pneumococcus.

2. The material obtained by this method is shown to consist mainly of a carbohydrate which appears to be a polysaccharide built up of glucose molecules.

3. Whether the soluble specific substance is actually the polysaccharide, or occurs merely associated with it, is still undecided, although the evidence points in the direction of the former possibility.

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IMMUNOLOGICAL RELATIONSHIPS OF CELL CONSTITUENTS OF PNEUMOCOCCUS.

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In the preceding paper (1) observations on the nature of the soluble specific substance of pneumococcus have been recorded. The present work concerns itself with the facts thus far ascertained in a comparative study of the chemical and immunological reactions of the protein[1] of pneumococcus. It seemed of interest to study the immunological relationship of the bacterial protein to the soluble specific substance of pneumococcus, and in the present paper certain differences in the serological specificity of the two classes of substances are brought out and related to their chemical nature. It would be beyond the scope of the present paper to attempt a review of the extensive literature on the subject of bacterial nucleoproteins in general, and this has already been done in reference books (2).

This work is presented, despite its incompleteness, because it points the way to a comparative study of the immunochemical relations existing between two different cellular constituents of the same organism. The first of these components, the so called soluble specific substance, has been discussed in the preceding paper; it need only be pointed out here that this reactive substance possesses none of the chemical properties of protein, that although antigenically it appears capable of stimulating little or no antibody response, serologically it exhibits to an extraordinary degree the reactions of type specificity in antipneumococcus sera. In other words, this non-protein constituent in isolated form is relatively and perhaps

[1] The word protein as used in this paper refers only to that portion of the dissolved pneumococcus cell precipitable in the cold by acetic acid and consisting mainly of nucleoprotein and mucoid.

absolutely inert as antigen, although it is highly reactive in the antibacterial serum of the homologous type of pneumococcus. On the other hand, by the methods described it is possible to separate from the bacterial cell another substance which is protein in nature and is also distinctive in its serological behavior from the soluble specific substance previously discussed.

Although the investigation of the relationship of chemical constitution to biologic specificity of pneumococcus is still in progress, the results thus far obtained are sufficiently interesting and perhaps significant enough to justify the presentation of facts already available.

EXPERIMENTAL.

Method.—The bacteria from actively growing broth cultures of pneumococci are recovered by centrifugation. The amount of material that can conveniently be worked up at any one time is, therefore, dependent on facilities for rapid centrifuging. In the present study broth cultures in units of from 6 to 12 liters have been treated in the following manner. The bacterial sediment, after removal of the culture fluid, is resuspended in about one-tenth volume of 0.85 per cent salt solution. To this suspension is added the minimum amount of bile necessary to effect solution of the bacterial cells. This procedure is carried out at ice box temperature (4°C.) in order to inhibit the possible proteolytic action of the intracellular enzymes which are released by lysis of the cells. Undissolved material is removed from the bile solution by centrifugation; the clear supernatant fluid containing the dissolved constituents of the cells is then treated by the method ordinarily employed for the isolation of the so called nucleoproteins from alkaline extracts of bacteria. The protein is precipitated from solution by the addition of dilute acetic acid (10 per cent). The acid is added slowly and the mixture carefully shaken; flocculation occurs promptly and is complete at a reaction faintly acid to litmus.

The precipitate obtained in this manner is separated by centrifugation and thoroughly washed two or three times in equal volumes of distilled water. The washed precipitate is redissolved in water by adding 0.1 N NaOH until the solution is faintly alkaline to litmus. This process of precipitation and washing is repeated at least three times. Before the last precipitation, the solution is passed through a Berkefeld filter V. The final precipitate is washed rapidly twice with acetone and once with dry ether and dried in a vacuum desiccator. The preparation obtained in this manner and referred to in the text as "protein" is a whitish, dry powder, readily soluble in faintly alkaline solutions.

Solutions of this substance give the usual qualitative color reactions characteristic of proteins of this nature: positive biuret, Hopkins-

Cole, Millon, xanthoproteic, and Molisch reactions. The hydrolyzed protein gives the purine reaction with Fehling's solution. The nitrogen content of a representative specimen of the protein was 16.0 per cent, the phosphorus content 0.5 per cent.

TABLE I.

Precipitin Reactions of Pneumococcus Protein (Type II) and Soluble Specific Substance (Type II).

Protein from Pneumococcus Type II.	Antipneumococcus serum.			Antityphoid Serum "T — A — B."	Normal horse serum.
	Type I.	Type II.	Type III.		
Lot 4, 1:1,000	++	++	++	—	—
" 5, 1:1,000	++±	++	++	—	—
Soluble specific substance of Pneumococcus Type II, 1:50,000.	—	++++	—	—	—

± indicates faint cloud; +, cloud; ++, marked cloud; +++, marked cloud with precipitation; and ++++, heavy precipitation, supernatant fluid clear.

TABLE II.

Precipitin Reactions of Pneumococcus Protein (Type II).

Protein from Pneumococcus Type II.	Antipneumococcus serum.			Normal horse serum.
	Type I.	Type II.	Type III.	
Lot 7, 1:200	++	++	++	—
1:400	++	++	++	—
1:800	++±	++	++	—
1:1,600	++±	+	+	—
1:3,200	++	+	+	—
1:6,400	+	±	±	—

Precipitin Reactions of Protein from Pneumococcus Type II in Antipneumococcus Sera Types I, II, and III.

Clear solutions of protein derived from Pneumococcus Type II were prepared by grinding weighed amounts of dry preparation with water, adding dropwise the minimum amount of 0.1 N sodium hydroxide to effect solution, and diluting to volume with salt solution. The antipneumococcus and antityphoid sera used were diag-

nostic sera prepared by immunization of horses with the respective bacteria. The reactions were carried out by adding increasing dilutions of protein solution in amounts of 0.5 cc. to an equal volume of serum so diluted that each 0.5 cc. contained 0.2 cc. of the original serum. All tests were incubated in the water bath at 37°C. and readings made at the end of the incubation period and after 18 hours at ice box temperature.

From Tables I and II it appears that solutions of protein prepared from one type of pneumococcus (Type II) react in about equal degree with all three types of antipneumococcus sera, and not with antityphoid or normal horse serum. The preparations of protein derived from Pneumococcus Type II by the method employed in the present investigation, therefore, seem to be species-specific rather than type-specific. This fact, if confirmed by subsequent investigation of the protein of pneumococci of other types, would indicate on the basis of specific precipitin reactions that all pneumococci possess in part a common basal specific protein. It must be borne in mind, however, that the immune sera used in these tests were not prepared by immunization with the isolated protein of the cell but by inoculation of horses with the intact bacteria. Immune sera are now being prepared from animals injected with purified protein as free as possible from other cell constituents such as the non-protein soluble substance. Until this is completed, it would, of course, be premature to attempt any interpretation of the significance of these reactions in terms of biologic specificity.

DISCUSSION.

It seems justifiable, then, on the grounds of the data presented in this and the preceding paper to conclude that the pneumococcus cell possesses at least two distinct substances which are intimately concerned with the biologic specificity of this organism. As a corollary of this, it would appear that pneumococcus immunity is related to two entirely distinct bacterial substances. One of these cellular constituents is the so called soluble specific substance, which as already pointed out is non-protein in nature and in its present state of purity is either carbohydrate or intimately associated with car-

bohydrate. This substance is chemically analogous to the bacterial gum which other investigators have isolated from various pathogenic and non-pathogenic encapsulated bacteria (3). However, previous studies have not related the chemical constitution of these polysaccharides to the serological or antigenic specificity of the organisms from which they were derived. The polysaccharide of pneumococcus, whether capsular substance or not, either is serologically reactive itself or is closely associated with some other substance which confers upon the organism the dominant character of type specificity. The protein fraction of the bacterial cell, on the other hand, is not type-specific but reacts in antipneumococcus serum regardless of type derivation. It is therefore species-specific, not type-specific.

The antigenic properties of these two substances; the relation of changes in specificity of pneumococcus to changes in virulence; and the relation of these substances to phenomena of infection and immunity are now being investigated.

SUMMARY.

1. The protein precipitated by acetic acid from solutions of pneumococci shows chemical reactions characteristic of nucleoprotein and mucoid.

2. The protein of pneumococcus, as contrasted with the non-protein soluble specific substance, exhibits species specificity rather than the type specificity characteristic of the latter.

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SURFACE TENSION OF SERUM.

VI. The Study of Immune Serum. Time-Drop and Initial Value of Surface Tension.

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Plate 11.

(Received for publication, March 2, 1923.)

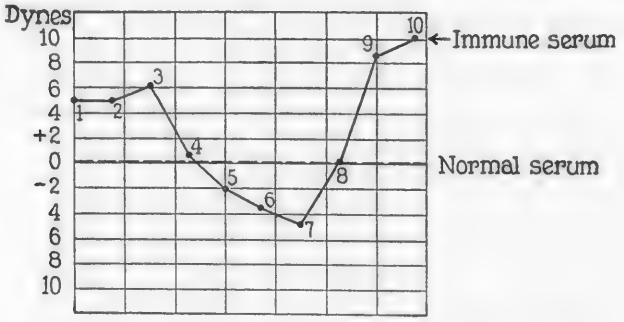
As stated in previous papers,[1-4] the four essential conditions under which the presence of antibodies in immune serum can be detected and followed by means of what might be termed the tensiometric method are: first, the dilution of the serum to 1:10,000; second, the use of strictly clean vessels; third, the care in handling the solutions, which must absolutely not be disturbed between the two measurements; and fourth, the study of the *time-drop*,[2] and not merely of the initial value of the surface tension. The first condition is a consequence of the numerous experiments reported in Papers I,[1] II,[2] IV,[3] and V.[4] The second has been emphasized in the same papers, and will be referred to again in the present paper. The third condition is satisfactorily fulfilled by the device represented in Fig. 1. However, the main object of this paper is to explain more extensively the reason of the fourth condition.

1. Time-Drop and Initial Value.—The results of a series of ten experiments on rabbits are reported in Text-fig. 1. From its mere inspection, it will be clear that the values of the initial surface tension of the solution of immune serum (1 part in volume of serum to 10,000 parts of saline 0.9 per cent) are sometimes higher and some-

[1] du Noüy, P. L., *J. Exp. Med.*, 1922, xxxv, 575.
[2] du Noüy, P. L., *J. Exp. Med.*, 1922, xxxv, 707.
[3] du Noüy, P. L., *J. Exp. Med.*, 1922, xxxvi, 547.
[4] du Noüy, P. L., *J. Exp. Med.*, 1923, xxxvii, 659.

times lower than the values of the solutions of normal serum. Hence, from these values alone, nothing could be gathered as to whether or not the serum contained antibodies. In order to make the chart clear, the initial value of the normal serum (before immunization) was taken as equal to 0, thus representing the axis of abscissæ, and the differences between the values of surface tension of the serum of the same animal, before and after immunization, were plotted with respect to this axis.

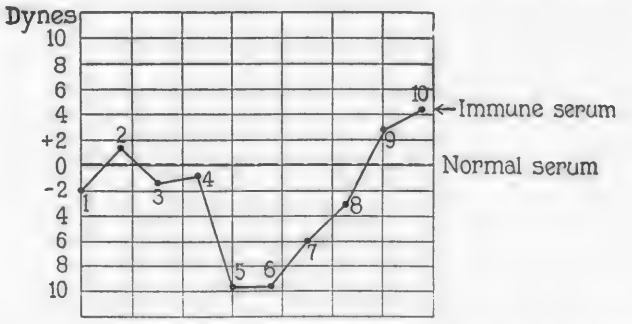
In Text-fig. 2, which represents the values of the surface tension of the solutions after standing for 2 hours, the same procedure was followed; *i.e.*, the values of the normal serum were taken as axis of 0,



TEXT-FIG. 1. Differences between the initial value of surface tension of normal and immune serum. Normal values are represented by the axis of abscissæ so that the differences are plotted in magnitude and sign.

and the differences between these values and those of the immune serum were plotted. For example, let us take two normal rabbits, A and B, whose serum solutions gave an initial value of surface tension of $A = 70.0$ dynes, and $B = 68.0$ dynes. After immunization, the initial value of the surface tension of the serum solution was $A_{imm.} = 73.0$ dynes, and $B_{imm.} = 66.0$ dynes. Then, if the normal values are used as the axis of abscissæ (ordinate = 0), the differences $A_{imm.} = +3.0$ dynes, and $B_{imm.} = -2.0$ dynes are plotted. In this way, the normal values will be represented by a straight line on both sides of which the differences between the values of the surface tension will be very obvious.

Consequently, if these two charts are brought together, however arbitrary the distance may be between the two parallel abscissæ representing the initial value of the normal serum, and the value after 2 hours, the ratio between their distance and the distance between the points representing the surface tension of immune serum in the two charts will express the ratio between the values of the so called time-drop in both cases, normal and immune serum. This was done in Text-fig. 3. It is clear that, in general, the variations in the value of the surface tension of immune serum after 2 hours



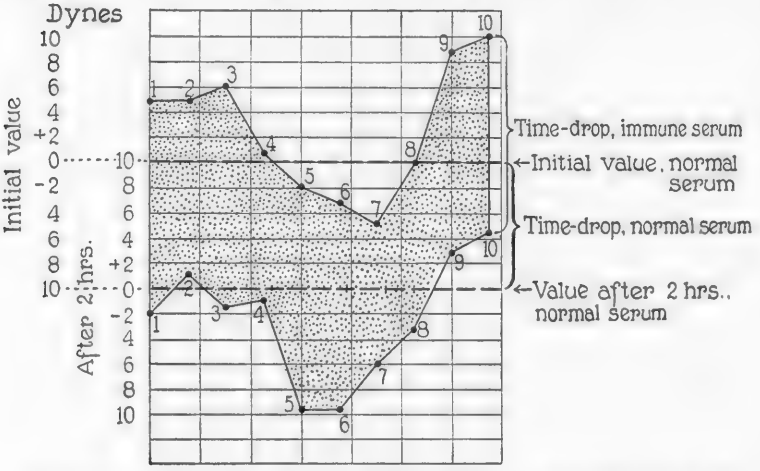
TEXT-FIG. 2. Differences between the values of surface tension of normal and immune serum after 2 hours. Normal values are represented by the axis of abscissæ so that the differences are plotted in magnitude and sign.

follow the fluctuations of the initial value more or less closely, so that, whether they are higher or lower, the time-drop is always greater for immune serum.

By joining the values of the surface tension of immune serum, two broken curves are obtained. The area between them may be integrated mechanically (Text-fig. 3, stippled area). By comparing the area thus measured (which is proportional to the time-drop for immune serum) to the area comprised between the two parallel lines (which is proportional to the time-drop of normal serum), a ratio is obtained from which the mean increase per cent in the time-drop of serum after immunization can be computed. In the present chart (Text-fig. 3), the increase is approximately 45 per cent. Now, all these experiments were by no means perfect; in other

words, they include measurements which were not made at the time when the maximum drop occurred, due to the presence of antibodies; *i.e.*, 13 days. Some were made too soon, some too late. Only two of them were made at the exact time, and they show an increase of 78 per cent (Nos. 3 and 5). A previous experiment[4] even showed an increase of over 100 per cent.

2. Normal Time-Drop.—Since the publication of the first paper on the action of antibodies on the time-drop, a number of data have been gathered concerning the time-drop of normal rabbit serum.



TEXT-FIG. 3. Text-figs. 1 and 2 brought together. The stippled area represents the time-drop of immune serum, in other words the differences between the initial value and the value after 2 hours, and the white area comprised between the two parallel lines corresponds to the time-drop of normal serum.

Comparison of the figures showed that, in the instances studied at least, the general value of the time-drop of the serum solution of a healthy, normal rabbit varied between 5 and 8 dynes in 2 hours. A higher drop, let us say above 10 dynes, always indicated that the rabbit was not absolutely normal. In that case, the increase in time-drop after immunization was smaller. Later, it was found that an epidemic of snuffles had developed in the cages in which the animals were kept. Subsequently, in order to obtain constant

results, all animals whose time-drop was above 10 dynes were discarded as not absolutely normal.

3. Details of the Technique.—It is necessary to lay particular emphasis on the cleaning of *all* vessels in which the serum, its solutions, and the distilled water are to be contained. They should be boiled for 2 hours in a concentrated solution of sulfuric acid, to which have been added 15 cc. of a saturated solution of potassium dichromate per liter. Just as important are the freshness and purity of the distilled water, and the cleanness of the sodium chloride to be used. Ordinary C.P. NaCl should be washed in a separating funnel. After 24 hours, the solution is collected from the bottom without stirring, a large amount of the supernatant liquid being left in the funnel. The solution is then recrystallized and used, great care being taken to prevent dust particles from falling on the crystals. The watch-glasses should be washed only 2 or 3 days at most before they are to be used; otherwise the liquid will not wet the glass perfectly. All the pipettes while in the hot cleaning solution must be washed inside by aspirating the acid up and down many times, by means of a rubber bulb and tube.

If this method is followed, the surface tension of the saline solution may be checked over and over again and will always give the same reading, within 0.05 dyne; otherwise, the results will vary greatly.

As a test for the cleanness of the vessels and the purity of the water and salt, allow the pure NaCl solution to crystallize; the crystals should be similar to the pictures published in Paper II, and should leave no rings on the glass.

As the position of the maximum time-drop in function of concentration obviously depends on the size of the watch-glass, that is on the ratio $\frac{\text{Total surface of liquid}}{\text{Volume of liquid}}$, it is important always to use the same size watch-glass and the same amount of liquid; for maximum drop at 10^{-4}, diameter of free surface of liquid equals approximately 4 cm. and volume of liquid equals approximately 2 cc.

CONCLUSIONS.

1. The initial surface tension of serum or serum solutions is not affected systematically by the presence of antibodies in the serum.

On the contrary, the time-drop in 2 hours is always increased, from 25 to 100 per cent.

2. It is extremely important, in order to demonstrate these phenomena, that the greatest care be taken regarding the cleanness of the vessels and the purity of water and NaCl.

3. It is equally important to use a device, for example such as is pictured in this paper, capable of preventing the jarring and shaking of the liquid.

4. The value of the time-drop of a normal, healthy serum is never higher than 10 dynes. Should it be higher, the serum must not be used for immunity experiments.

EXPLANATION OF PLATE 11.

FIG. 1. Turntable on ball bearings used in connection with the tensiometer to prevent jarring of the solution. The watch-glasses are brought successively under the platinum loop and raised by means of a screw.

FIG. 1.

(du Noüy: Surface tension of serum. VI.)

THE INFLUENCE OF THE SUPRARENAL CORTEX ON THE GONADS OF RABBITS.

I. The Effects of Suprarenal Injury (by Removal or Freezing) on the Interstitial Cells of the Ovary.

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(Received for publication, February 26, 1923.)

INTRODUCTION.

It is well known that hypertrophic changes occur in the interstitial cells of the ovary, and in the suprarenal cortex, in certain animals during pregnancy. Likewise, investigators are in general agreement that removal of the gonads causes hypertrophy of the suprarenal cortex. With these facts in mind, it occurred to us that conversely suprarenal injury might produce changes in the sex glands. We therefore studied the data of nearly 400 rabbits that had been subjected to various types of suprarenal injury. In the present communication the details of the observations on the changes which take place in the ovary following suprarenal injury are reported. Brief references to certain of these changes have already been made by one of us (1, 2) in other publications.

LITERATURE.

While the literature contains several references dealing directly with the effects of gonadectomy and pregnancy on the suprarenal cortex, there seem to be no references dealing particularly with the effects of suprarenalectomy on the gonads and only a few in which such a relation is considered at all.

Novak (3) studied the effects of suprarenalectomy on the genital tracts of male and female white rats. The animals survived the operation, done in two stages, from several days to several months. He noted that suprarenal extirpa-

tion caused hypoplasia or atrophy of the genital tract. He did not study particularly the effects on the interstitial cells either of the testis or of the ovary.

Cesa-Bianchi (4) described fatty degeneration of the interstitial cells of the ovary occurring in guinea pigs which survived double suprarenalectomy for from 2 to 4 days, but the functional significance of such changes in lipoid-rich cells and especially when occurring in moribund animals is doubtful. Likewise the observation of Sserdjukoff (5) on four pregnant cats, all of which died within 3 days after suprarenalectomy and in which an increase was found in the size of the interstitial and lutein cells, is open to the severe criticisms that only four animals were used; that pregnancy normally induces hypertrophy of the interstitial cells; and that animals moribund from the time of operation could show hypertrophic changes ascribable to suprarenal insufficiency. Pende (6), working with cats, also reported an increase in the interstitial cells of the ovary following suprarenalectomy. But Elliott (7) reports that he did not observe any significant changes in the ovaries or testes of cats following partial or double suprarenalectomy.

On the other hand, there are many observations showing that gonadectomy causes enlargement of the suprarenal cortex in rabbits, guinea pigs, and dogs (Theodossieff (8), Schenk (9), and Kolde (10)), although Elliott (7) was unable to detect any enlargement of the suprarenal glands in twelve gonadectomized cats. Likewise, many observers have noted the enlargement of the cortex in pregnancy (Guieysse (11), Kolmer (12), Kolde (10), and Schenk (9)).

Riddle (13) recently reported enlargement of the suprarenals in pigeons during ovulation.

Embryology.

The interstitial cells of the ovary were first described by Pflüger in 1863, but His (14) in 1865 was the first to describe them in detail in the ovary of the cat and to discuss their possible significance. Their occurrence in close association with the spindle-shaped stroma cells, and in apparently close association with the blood capillary network led him to regard them as developed from stroma cells, but he also suggested that they might arise from cells embryologically related to blood capillaries.

An association of the interstitial cells with the theca interna of follicles was described almost from the time of their first observation. His believed that the interstitial cells gave rise to the theca interna, while more recent investigators have claimed that the interstitial cells were derived from the theca interna cells of the immature follicles undergoing regression or atresia (von Koelliker (15), Allen (16), Fellner (17), and Kingsbury (18)).

Other modes of origin of the interstitial cells have been advanced. Lane-Claypon (19) contends that in the rabbit ovary they are derived from the germinal epithelium as cell cords in common with the ova, and that the interstitial cells remain grouped together in spaces between the follicles instead of arranging themselves around developing ova. She also believes that ova may be formed

from interstitial cells and that this actually begins to take place in the maternal ovary on about the 20th day of pregnancy.

We have briefly stated the two principal views concerning the origin of the interstitial cells of the ovary, and it is interesting to note in this connection that there are similarly two principal views regarding the origin of the interstitial cells of the testes: (1) that they are connective tissue cells, and (2) that they are epithelial cells derived from the germinal epithelium.

The relation between the interstitial and lutein cells is found in the view more or less definitely expressed by many workers that no fundamental differences exist between the regressive changes that occur in corpora lutea and those occurring in unruptured follicles undergoing degeneration. Schulin (20) was among the first to express this view, and he believed that he detected intermediate forms between typical atresia of the follicles and corpus luteum formation. Many others have expressed their belief in the fundamental similarity of the two processes.

Allen, who believes in the connective tissue origin of the interstitial cells, states that the interstitial and lutein cells look so much alike that it seems improbable that two groups of cells morphologically identical could have arisen from such diverse elements as connective tissue of the theca interna on the one hand, and granulosa cells on the other. He favors a common origin for these two groups of cells, believing with Clark (21), Van Beneden (22), and von Koelliker that the lutein cells and interstitial cells arise from the theca interna. Others, who accept the common origin of the interstitial and lutein cells, believe, however, that they both arise from germinal epithelium. Van der Stricht (23), working with the bat, found that both granulosa and interstitial cells of the theca interna take part in the formation of the corpus luteum. However, the similarity of the structures arising from both ruptured and unruptured follicles and the embryological relation between the interstitial and lutein cells is emphatically denied by many, particularly Sabotta (24).

The significance of the common origin of these different ovarian elements becomes more apparent when the views regarding the functional importance of these cells are considered. The conception of a common origin of follicle and interstitial cells permits of a functional association between interstitial and lutein cells. It allows a satisfactory answer to the perplexing fact that interstitial cells have not been definitely found in many species. Even in the human ovary, while they have been apparently demonstrated by some workers, their existence has been questioned by others. In any event, they are few in number and require a special technique for demonstration.

This brief summary of the conflicting views concerning the relation between interstitial and lutein cells indicates that anatomical studies must be combined with carefully planned physiological and chemical studies in order to determine whether the lutein cells and interstitial

cells have a common origin either from the germinal epithelium or from the connective tissue, or whether the lutein cells arise from one type of tissue and the interstitial cells from the other.

Histology.

The interstitial cells of the rabbit ovary are large polyhedral cells about 15 micra in diameter. The nucleus is large, vesicular, and distinctly polychromatic, while the cytoplasm is crowded with droplets of lipoid substance. The chemical composition of the lipoid substances has not been definitely determined, but like the lipoids of the suprarenals they contain cholesterol and lecithin. Cowdry (25) could not confirm the observations that the mitochondria exhibit special peculiarities. The reticular apparatus has been carefully studied without bringing to light any special distinctive features. Pigments other than lipochrome are of rare occurrence.

A number of writers have spoken of the interstitial cells of the ovary as the interstitial gland. Bouin (26) and Limon (27) were among the first to do so. The view is based largely on morphological studies of the interstitial cells in the rabbit and bat. Interstitial cells are present in varying numbers in different animals. They are present in large numbers in the rabbit, bat, and cat ovaries, scantily developed in the human ovary, and up to the present time have not been demonstrated in the pig, sheep, and cow.

Presentation of Data.

This report is based on a study of the clinical and necropsy protocols of thirty rabbits that were subjected to double suprarenalectomy, and of thirteen rabbits in which the suprarenals were injured by partial removal or by freezing.[1] All animals were sexually mature at the time of death. The principal data of these two groups have been collected in Tables I and II and arranged according to the duration of life after suprarenalectomy or injury.

Double Suprarenalectomy.—Examination of Table I shows that bilaterally suprarenalectomized rabbits which survive the operation for several weeks frequently show enlargement of the ovaries, while

[1] All operations were performed under ether anesthesia.

those succumbing within 2 weeks generally do not. A survival period of 30 days has been taken arbitrarily as sufficient time in which cellular changes or compensatory hypertrophy would manifest themselves.

Thirteen animals survived the operation for less than 30 days and of those, ten, or 77 per cent, showed small, normal sized ovaries, with no significant hypertrophy of the interstitial cells. Three, or 23 per cent, presented enlarged ovaries. These three (Nos. 8, 12, and 13) survived double suprarenalectomy for 17, 24, and 27 days respectively and died with symptoms of suprarenal insufficiency.

Of the seventeen rabbits which survived double suprarenalectomy for more than 30 days, four, or 24 per cent, showed no ovarian enlargement, eight, or 47 per cent, showed moderately enlarged ovaries (about twice normal size), while five, or 29 per cent, showed markedly hypertrophied ovaries (about three times normal size). The enlargement consists essentially of an hypertrophy of the interstitial cells. The enlarged cells frequently measure 30 micra in diameter. They become very fatty and often take on a radial arrangement similar to that seen in the suprarenal cortex. That there exists a striking general relationship between the degree and frequency of ovarian hypertrophy and the duration of life after suprarenalectomy is shown by the fact that six of the seven animals surviving over 60 days showed ovarian enlargement, while seven of the ten surviving between 30 and 60 days showed enlargement, and only three of the thirteen surviving less than 30 days showed enlargement.

Further analysis of the data shows that, in addition to the relation between the duration of life and the degree of ovarian hypertrophy, there is a general parellelism between the amount of accessory suprarenal tissue found at autopsy and the degree of ovarian enlargement. In the first instance, this adds further data that survival after double suprarenalectomy depends on the presence of accessory suprarenal cortex, and secondly, it suggests that both the hypertrophy of the accessory cortical tissue and that of the interstitial cells of the ovary are compensatory and brought about in part at least by the same mechanism.

Partial Suprarenalectomy.—The effects on the ovaries of partial suprarenalectomy by removal or freezing have been studied in thirteen rabbits. The data relative to these are given in Table II. In twelve

TABLE I.

Double Suprarenalectomy.

Rabbit No.	Interval between removal of right and left suprarenals.	Major cause of death.	Interval between suprarenalectomy and examination of ovaries.	Fragments or accessory suprarenals.	Size of ovaries.	Interstitial cells.	Interval between last pregnancy and examination of ovaries.	Corpora lutea.
	days		*days*				*days*	
1	7	Pneumonia.	1	None found.	Normal.	Normal.	60	
2	12	Suprarenal insufficiency; infected right suprarenalectomy wound.	2	" "	"	"	213	
3	148	Suprarenal insufficiency.	2	One accessory.	"	"		
4	0	Pneumonia; snuffles.	2	Two 1 mm. accessories.	"	"		
5	0	"	2	None found.	"	"		
6	14	Suprarenal insufficiency; wound infection.	7	" "	"	"		
7	9	Suprarenal insufficiency.	10	" "	"	Moderately hypertrophic.		
8	215	" "	17	" "	Markedly enlarged.	Markedly hypertrophic.		
9	95	" "	17	One small accessory.	Normal.	Normal.		Few small groups of lutein cells.
10	25	Snuffles; suprarenal insufficiency.	17	None found.	"	"		

11	37	Pneumonia.	20	One small accessory.	Normal.	Moderately hypertrophic.		
12	0	Sacrificed.	24	None found.	Markedly enlarged.	Markedly hypertrophic.		
13	37	Suprarenal insufficiency.	27	" "	Moderately enlarged.	Moderately hypertrophic.	80	
14	7	" " respiratory infection.	30	Six small accessories.	Moderately enlarged.	Moderately hypertrophic.		
15	7	Suprarenal insufficiency.	30	Two small accessories.	Moderately enlarged.	Moderately hypertrophic.		
16	68	" " hastened by anesthetic.	30	One minute accessory.	Markedly enlarged.	Markedly hypertrophic.		Several large corpora lutea; uterus normal; mammary gland hypertrophic.
17	0	Abscess in neck; sacrificed.	32	None found.	Normal.	Normal.		Few small groups of lutein cells.
18	143	Arsenic poisoning.	34	" "	"	"	195	
19	7	Died after exploratory operation.	35	Two small accessories; one fragment 1.5 by 1 mm.	"	"		
20	75	Empyema.	40	Two small accessories.	Moderately enlarged.	Markedly hypertrophic.		
21	32	Suprarenal insufficiency.	42	None found.	Markedly enlarged.	Markedly hypertrophic.		
22	68	Snuffles; sacrificed.	43	One 0.5 mm. stump on left; one 4 by 3 mm. stump on right; one small accessory.	Moderately enlarged.	Moderately hypertrophic.		
23	21	Suprarenal insufficiency.	47	Three small accessories.	Moderately enlarged.	Markedly hypertrophic.		

TABLE I—*Concluded.*

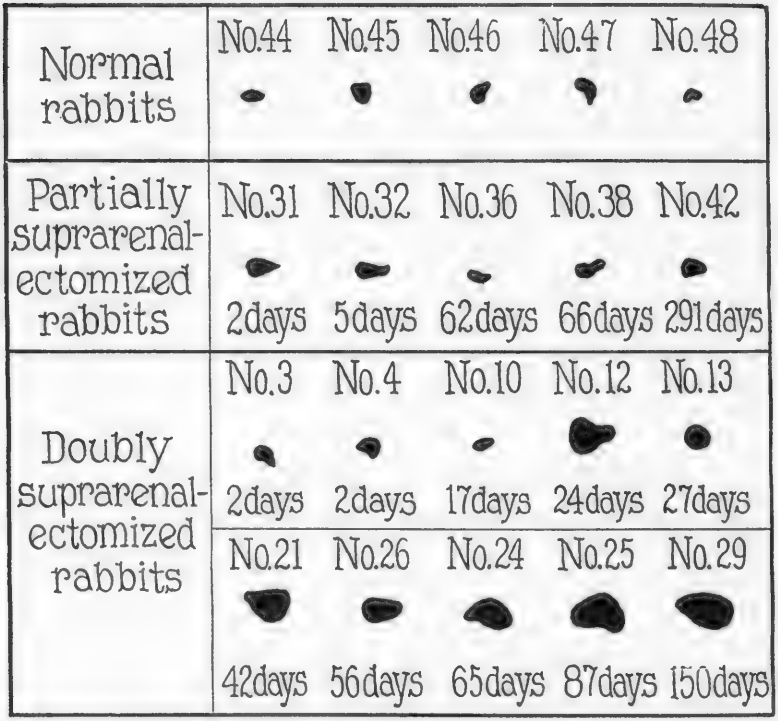
Rabbit No.	Interval between removal of right and left suprarenals.	Major cause of death.	Interval between suprarenalectomy and examination of ovaries.	Fragments or accessory suprarenals.	Size of ovaries.	Interstitial cells.	Interval between last pregnancy and examination of ovaries.	Corpora lutea.
	days		*days*				*days*	
24	180	Subcutaneous abscesses; bronchopneumonia; sacrificed.	65	Mass of hyperplastic cortex with some medulla on right.	Markedly enlarged.	Markedly hypertrophic.		Few small groups of lutein cells.
25	6	Sacrificed to terminate experiment.	87	One large accessory 5 by 2.5 mm.	Markedly enlarged.	Markedly hypertrophic.		
26	6	Sacrificed to terminate experiment.	88	One very large accessory.	Moderately enlarged.	Moderately hypertrophic.	53	
27	21	Strong and healthy; sacrificed.	102	Three small accessories.	Normal.	Normal.		
28	0	Moribund; sacrificed.	143	One very large accessory.	Moderately enlarged.	Markedly hypertrophic.		
29	25	Intravenous injection of vaccine.	150	Two small accessories.	Markedly enlarged.	Markedly hypertrophic.		Several large corpora lutea; uterus normal; mammary gland hypertrophic.
30	100	Suprarenal insufficiency.	150	One small accessory.	Moderately enlarged.	Markedly hypertrophic.	36	

TABLE II.

Partial Suprarenalectomy.

Rabbit No.	Major cause of death.	Interval between suprarenalectomy and examination of ovaries.	Size of ovaries.	Interstitial cells.	Interval between last pregnancy and examination of ovaries.	Corpora lutea.
		days			*days*	
31	Snuffles; chronic pneumonia.	2	Normal.	Normal.	256	
32	Anesthetic.	5	"	Moderately hypertrophic.		
33	Infection of wound.	8	"	Normal.	138	
34	Extreme emaciation, mange, and right purulent pleurisy; sacrificed.	11	"	"	190	Several small groups of lutein cells.
35	Killed by accident.	47	"	"		
36	Pneumonia.	62	"	"		
37	?	65	"	"		
38	Sacrificed.	66	"	"		
39	Marked emaciation; weakness; sacrificed.	150	"	"	153	
40	Mange and weakness; sacrificed.	159	"	"	127	
41	" arsenic poisoning.	174	"	"	199	Few small groups of lutein cells.
42	Myxedema; mange; sacrificed.	291	"	"		
43	Paralysis of hind legs; peritonitis.	1,095	Markedly enlarged.	"	532	

the ovaries were of average normal size, and the interstitial cells generally showed no recognizable differences from those seen in normal rabbits. In one very old animal (No. 43) known to have been pregnant four times, the ovaries were much enlarged. The interstitial cells, however, were not prominent, and the enlargement was found to be due to a considerable increase in the stroma tissue.



TEXT-FIG. 1. Tracings of representative cross-sections of ovaries of normal rabbits, partially suprarenalectomized rabbits, and doubly suprarenalectomized rabbits, showing the relation of ovarian hypertrophy to duration of life after suprarenalectomy.

As this study was incidental to the main purpose for which these rabbits were originally used, the weights of the ovaries were not recorded. All autopsies were made by one of us, and in most instances

transverse sections were taken from the middle third of the ovary for microscopic study. Formalin fixation, celloidin embedding, and hematoxylin and eosin staining were used in all. Tracings of the actual size of cross-sections of the ovaries are given in Text-fig. 1. These are arranged to show the size of the normal ovary and the size of the ovary after partial and after double suprarenalectomy in relation to duration of life.

Three of the rabbits, though not pregnant, were found to have large corpora lutea, similar in all respects to those of pregnancy. This observation raises several important questions in relation to ovulation in rabbits (28) and the influence of suprarenalectomy on corpus luteum production, which will be dealt with in a separate paper.

Nineteen additional experiments in which double or partial suprarenalectomy was complicated with pregnancy were not included in the present report.

The work of previous observers was confirmed in that no evidence of variation in the size or structure of the ovary dependent upon season or rut was found (29).

DISCUSSION.

Data have been presented concerning thirty rabbits that were subjected to double suprarenalectomy, and thirteen in which the suprarenals were injured by partial removal or by freezing. Of the seventeen animals surviving double suprarenalectomy for over 30 days, thirteen, or 76 per cent, showed either moderate or marked enlargement of the ovaries, while of the thirteen that survived this procedure for less than 30 days, only three, or 23 per cent, showed enlarged ovaries. These (Nos. 8, 12, and 13) survived suprarenalectomy 17, 24, and 27 days respectively, and represent, we believe, the lower limits as regards time in which obvious ovarian enlargement can take place in the presence of high grade suprarenal insufficiency. This enlargement consists essentially of an hypertrophy of the interstitial cells.

Of the rabbits in which the effects of partial suprarenalectomy were studied, twelve of the thirteen presented normal sized ovaries.

While the degree and frequency of ovarian hypertrophy following double suprarenalectomy cannot be brought into mathematical relation

with the duration of life after double suprarenalectomy, still there is a striking general relation.

Nineteen additional experiments in which double or partial suprarenalectomy was complicated by pregnancy were not included because it is well known that hypertrophy of the interstitial cells occurs during pregnancy. Lane-Claypon, studying a large number of rabbits, gives 17 micra as the average diameter of the interstitial cells of normal adult ovaries. During pregnancy these cells enlarge and reach a maximum of 32 micra on the 22nd day. Shortly after pregnancy the ovary undergoes regression, reaching its former size within 2 months. The ovarian enlargement observed in the present study following double suprarenalectomy could not be attributed to previous pregnancies as many of the animals were known never to have been pregnant, and, in addition, the list of partially suprarenalectomized rabbits includes a number of animals that were pregnant several times but in which the ovaries were of normal size at autopsy. This confirms the work of others with regard to regression of the ovary following pregnancy. Functional enlargement of the ovaries also occurs in other associations than pregnancy and suprarenal injury. It has been shown that compensatory hypertrophy follows partial castration (30), and that the hypertrophy is approximately proportional to the amount of ovarian tissue removed. There is also evidence that the interstitial cells of the ovary enlarge with the suprarenal cortex in chronic infections in rabbits.

As to the fate of the interstitial cells, Kingsbury (18) confirms the conclusions of Saimont and others that they are transient structures. He worked on cats. He further agrees with Saimont that their fate is not one of degeneration but that they revert back to the cell type from which they developed, which he believes is the stroma cell. However, the belief that they may develop from and revert back to a type morphologically resembling stroma cells is sufficient evidence that they are highly specialized cells.

The work reported here offers additional physiological evidence for the interrelation that has been suspected for some time from clinical observation. Most of the previous experimental work attempting to clarify the problem of suprarenal-gonadal interrelation has dealt with the effects of pregnancy, castration, and ovulation on the suprarenal cortex. The mechanism of the production of ovarian hypertrophy

following double suprarenalectomy is not understood. A plausible interpretation is that the enlargement is compensatory and in support of this are the facts that removal of the gonads, pregnancy, and ovulation also induce an hypertrophy of the suprarenal cortex. These facts clearly indicate an apparent reciprocal relation between these tissues.

CONCLUSIONS.

1. Moderate or marked ovarian enlargement has been observed in 76 per cent of rabbits which survived double suprarenalectomy over 30 days.

2. The ovarian enlargement consists essentially of an hypertrophy of the interstitial cells.

3. The hypertrophy of the interstitial cells is a compensatory reaction and adds further data to the functional interrelation between the suprarenal cortex and the interstitial cells.

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THE INFLUENCE OF THE SUPRARENAL CORTEX ON THE GONADS OF RABBITS.

II. The Effects of Suprarenal Injury (by Removal or Freezing) on the Tubules and Interstitial Cells (Leydig) of the Testis.

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In a previous paper (1) it was shown that, of seventeen female rabbits which survived double suprarenalectomy for a month or more, striking enlargement of the ovaries was observed in thirteen, or 76 per cent, while of the thirteen rabbits with partial suprarenalectomy, only one showed enlargement of the ovaries. This enlargement consists essentially of an hypertrophy of the interstitial cells.

In view of the fact that enlargement of the suprarenal cortex usually occurs in rabbits and guinea pigs following gonadectomy and also in these animals during pregnancy, and in birds during ovulation, there seems to be some reciprocal relation between the ovary, particularly its interstitial cells, and the suprarenal cortex. It was suggested that the hypertrophy of the interstitial cells following suprarenalectomy may be of the nature of a compensatory hypertrophy.

The observations regarding the ovarian changes prompted us to study the testes of rabbits which survived double or partial suprarenalectomy with particular reference to the cells of Leydig.

LITERATURE.

Little has been published concerning the effects of suprarenalectomy on the gonads. Novak (2) studied the effects of suprarenalectomy on the genital tracts of male and female white rats. He removed the second suprarenals 7 to 14 days after the first. The period of survival after suprarenalectomy varied from several days to months. In some animals there were no effects, particularly in adult

males, and he ascribed this to the well known fact that accessories are found in a large percentage of white rats. He concluded that suprarenal extirpation in white rats induces a hypoplasia or atrophy of the genital tracts that is more pronounced the younger the animal is at the time of operation. Partial suprarenalectomy was without effect. He believed that the main cause of the genital atrophy was the removal of the internal secretion of the suprarenals. Novak, however, did not study particularly the interstitial cells either of the testis or of the ovary.

Elliott (3) stated that he did not observe any significant changes in the ovary or testis of cats following partial or double suprarenalectomy.

The work of Cesa-Bianchi (4) and Pende (5) dealt only with the effects of suprarenalectomy on the ovary.

Hewer (6) fed white rats with the dried cortex of ox suprarenals and injected both lipoid and non-lipoid extracts. Her work confirms the observations of others that lipoid extracts of the suprarenal cortex cause rats to become extremely fat. In addition, she reported changes in the testes, but they were not of a specific nature.

Recently Leupold (7) attempted to establish a relation between the suprarenal and the testis, mainly on morphological and microchemical grounds. He states that in the male, during sexual activity, the doubly refractive substances and cholesterol of the suprarenals increase. During the periods of sexual quiescence there is a diminution in the cholesterol content. In atrophic testes, cholesterol esters appear in large amounts in the Leydig cells; Leupold believes that these cells resorb the liberated lipoids from the disintegrated tubular epithelium.

In another paper (8) Leupold discusses cholesterol metabolism in relation to spermatogenesis based on his morphological and chemical studies of human autopsy material. He points out that a diminution in lipoid content of the suprarenal cortex is associated with a similar diminution of the lipoids in the interstitial cells. The reverse was also found, large amounts of lipoid in the suprarenal being associated with large amounts in the interstitial cells.

Leupold attempted to reinforce his morphological studies on the close lipoidal relation between the cells of Leydig and those of the suprarenal cortex by animal experimentation. He carried out unilateral suprarenalectomy on three cats and double suprarenalectomy on one and examined the testes within 8 days after operation. Extensive destruction of the spermatogenic cells was found. From this he concluded that the cholesterol esters of the suprarenal cortex have a protective influence on the sex glands in that they bind toxins which would otherwise destroy these cells, and secondly, that they have a regulatory function on the amount of these esters in the Leydig cells. He supports the old conception that Leydig cells supply the lipoids as foodstuffs to the sperm cells.

However, Leupold's conclusions are based primarily on human autopsy findings and his elaborate hypotheses have not been supported by adequate experimental work.

With regard to the effects of gonadectomy on the suprarenal cortex there are several observations showing that this procedure causes an enlargement of the cortex in rabbits, guinea pigs, and dogs (Theodossieff (9), Schenk (10), and Kolde (11)).

Embryology.

Embryologically, the testis, ovary, and cortex of the suprarenal arise from a common anlage, the urogenital fold, or Wolffian ridge. The vertebrate embryo forms at first an indifferent reproductive gland. The newly formed "testes" or "ovaries" are morphologically alike up to a certain period of development, but later by emphasis of certain characters the sexually differentiated organ is formed. It has been shown for all classes of vertebrates, with the exception as yet of mammals, that the first genital cells have a special origin, probably being derived directly from the segmentation cell. Felix (12), therefore, terms these cells "primary genital cells" in contradistinction to those that are differentiated from the epithelial covering of the reproductive gland, which he terms "secondary genital cells."

Concerning the origin of the cells of Leydig, there are two principal views. According to one they arise from the connective tissue, and according to the other they are epithelial cells derived from the germinal epithelium. Allen (13) and Whitehead (14) have made the most recent studies on the embryology of the cells of Leydig and support the view that they are of connective tissue origin.

Presentation of Data.

This study of the effects of suprarenalectomy on the testis is based on the examination of the gross and microscopic findings in suprarenalectomized rabbits.[1] The tissues were fixed in 10 per cent formalin, embedded in celloidin, and stained with hematoxylin and eosin.

The testes of 48 rabbits subjected to double suprarenalectomy and 45 subjected to partial suprarenalectomy were studied.

Double Suprarenalectomy.—The periods of survival of the 48 rabbits in which bilateral suprarenalectomy was carried out, are as follows:

Rabbits surviving from 1 to 15 days	12
Rabbits surviving from 16 to 29 days	9
Rabbits surviving from 30 to 60 days	9
Rabbits surviving from 61 to 90 days	6
Rabbits surviving longer than 90 days	12

[1] All operations were performed under ether anesthesia.

Of the twenty-seven rabbits surviving double suprarenalectomy for more than 30 days,[2] twenty-two showed normal testes microscopically. Of these, three were reported atrophic in the gross, but this apparent atrophy could not be confirmed microscopically.

In this connection it is of interest to recall the conclusions of workers who attempted to correlate the gross and microscopic appearances of testes of men dying of general diseases. Berberich and Jaffé (15) recently studied the testes of 100 human cases in which death was due to general diseases and they came to the conclusion that, with the exception of marked changes, gross findings do not permit of a final interpretation as to the condition of the organ. Simmonds (16) was of the same opinion. Our own experience in a series of 224 patients dying of chronic diseases is in harmony with the expressed view.

The twenty-two rabbits with normal testes microscopically showed well preserved tubules, with normal active spermatogenic elements. The intertubular connective tissue was not increased and there were very few Leydig cells, which were mainly disposed about small blood vessels. The tunica and rete showed no recognizable deviation from the normal. The veins were frequently congested, this being occasionally quite pronounced in animals dying of acute suprarenal insufficiency, with marked cardiovascular asthenia. Two of twenty-seven rabbits, one surviving 41 and the other 115 days after double suprarenalectomy, showed a slight increase in the number of Leydig cells, without any detectable variations in the other elements of the testis. This slight increase may be relative, but if absolute it could be attributed, we believe, to physiological variations rather than to the effects of extirpation of the suprarenal glands. Both rabbits were adults when the second suprarenals were removed.

Three of the twenty-seven rabbits showed atrophic changes in the tubules without definite changes in the Leydig cells. In one animal

[2] As pointed out many times, accessory cortical tissue is probably present in all rabbits and can be readily demonstrated in about 70 per cent. A larger number of males survive double suprarenalectomy than of females. This is due, we believe, to the more frequent occurrence of accessory cortex which for the most part is found along the spermatic cord. However, skilled surgical technique and careful postoperative care are important factors in survival.

which lived 51 days after removal of the second suprarenal, no apparent cause for the atrophy could be discovered. It was an adult when the second suprarenal was removed. Two other animals, one surviving 35, the other 65 days following removal of the second suprarenals, also showed atrophy of the testes with no changes in the Leydig cells. A possible cause for the atrophy was complete thyroidectomy performed in each case 23 days prior to the removal of the second suprarenal.

Twenty-one rabbits survived suprarenalectomy for less than 30 days. Of these, twelve had normal tubules and Leydig cells, five had atrophic tubules with normal Leydig cells, and one had atrophic tubules with a slight relative increase in the interstitial cells. Of the six rabbits with atrophic tubules, in one no apparent cause for the atrophy could be determined; two were very mangy and weak throughout the period of experimentation; one had paralysis of the hind legs and incontinence during the last month of life; and two had been subjected to thyroidectomy in addition to suprarenalectomy.

Three showed a slight relative or absolute increase in the interstitial cells with normal tubules.

The condition of the interstitial cells and tubules in the 48 bilaterally suprarenalectomized rabbits is summarized as follows:

Condition.	Rabbits surviving over 30 days.	Rabbits surviving less than 30 days.	Per cent.
Normal tubules and Leydig cells	21	12	71
Atrophic " " normal Leydig cells	4	5	17
" " " increased " "		1	2
Normal " " " " "	2	3	10

Thus the tubules were found to be normal in 81 per cent, while the Leydig cells were unchanged in 88 per cent.

Partial Suprarenalectomy.—45 animals in which the suprarenals were injured by partial removal or by freezing were studied. Seven were eliminated because the testes were not completely developed. Partial suprarenalectomy, however, apparently does not prevent or delay the normal development of the testes as many animals in this group were partially suprarenalectomized before sexual maturity.

Seven other rabbits were eliminated because unilateral or bilateral vasoligature complicated partial suprarenalectomy, although in several instances no effects were observed as a result of this procedure. In one rabbit, vasoligature was performed when the animal was 1 month old; when the testes were examined at 7 months they were fully differentiated. Of the remaining thirty-one rabbits, twenty-six, or 83 per cent, showed normal tubules and interstitial cells; two, or 7 per cent, showed normal tubules with a slight relative or absolute increase in the cells of Leydig; three, or 10 per cent, showed atrophic tubules and in one of these it was associated with a relative increase in the interstitial cells.

DISCUSSION.

Data have been presented concerning 48 rabbits that were subjected to double suprarenalectomy, and 45 in which the suprarenals were injured by partial removal or freezing. In the bilaterally suprarenalectomized rabbits 81 per cent presented microscopically normal seminiferous tubules, and 88 per cent had normal Leydig cells. Partial suprarenalectomy also was without specific effect on the testes. Of those analyzed, 90 per cent showed normal tubules, and 86 per cent showed no change from the normal in the cells of Leydig.

In the few instances in which atrophy of the testes was noted, it was believed that long standing illness, progressive cachexia, or some additional operative procedure, as for instance thyroidectomy, offered a more likely explanation than injury of the suprarenals.

That long standing illness in man has an unfavorable effect upon the testes has been known for some time. Cordes (17) found in chronic diseases an increase in connective tissue, and he noted that this increase was most pronounced in cachectic states. In addition, he reported shrinking of the tubules, hyaline thickening of their walls, and impairment of spermatogenesis. The cells of Leydig showed no constant change, sometimes showing an increase and at other times remaining unchanged. Hansemann (18), Thaler (19), and Berberich and Jaffé (15) have come to similar conclusions.

In an unpublished study of the testes of 224 patients dying of chronic diseases at Montefiore Hospital we have found that fre-

quently these organs showed pronounced injury. The tubules were shrunken, the walls showed hyaline thickening, and spermatogenesis was often impaired, the sperm cells frequently disappearing entirely and leaving only a row of Sertoli cells lining the lumen. The intertubular connective tissue was generally increased. The Leydig cells showed no constant changes. They were relatively increased in about 25 per cent of the cases, but much more frequently they showed no change.

We believe that such factors influenced Novak's results. Double suprarenalectomy in rats frequently induces no serious insufficiency. The hypoplasia or atrophy of the genital tracts reported by Novak was probably brought about by complicating factors.

We were particularly interested in the interstitial cells of Leydig and were struck by the great difference in behavior to double suprarenalectomy between these cells and the interstitial cells of the ovary. Even prior to their discovery in 1850, Berthold (20) was speculating on the dual function of the testis. But since then, in spite of the fact that a large amount of work has been done in attempting to discover and evaluate the internal secretory function of the various constituents of the testis, the complexity of the organ has made it impossible to assign with certainty definite secretory activities to its different components. Transplantation, traumatism, vasoligature, cord ligature, induced cryptorchidism, partial removal, exposure to x-rays and radium, and freezing have all been unsuccessful in dissociating the spermatogenic, Sertoli, and Leydig cells, and for this reason workers have been unable to obtain unequivocal evidence of specific internal secretory activity of any of the elements of the testis, or to extend the observations of Brown-Séquard and his followers on the effects of extracts. That the organ has an important function in addition to the production of the sperm cells there is no doubt. The effects of castration before puberty clearly demonstrate the profound physiological disturbance in the mental and physical development induced by this procedure.

Many have considered that the cells of Leydig and the interstitial cells of the ovary are homologous. The evidence in favor of this view is based on morphological and microchemical studies. More detailed studies have shown that there are sufficient histological

differences between the two to warrant considerable hesitation in accepting this view. The fact that the interstitial cells of the ovary readily undergo hypertrophy after double suprarenalectomy in rabbits while the cells of Leydig show no such response in this animal we have interpreted as physiological and biological evidence against their functional homodynamy.

CONCLUSIONS.

1. Double or partial suprarenalectomy in rabbits produces no specific changes either in the tubules or in the interstitial cells of the testes.

2. This is in sharp contrast with the marked hypertrophy of the interstitial cells of the ovary which usually occurs in rabbits surviving double suprarenalectomy 30 days or more.

3. These facts indicate that the interstitial cells of the testis and ovary are not functionally homologous.

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